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Regulation of Fatty Acid Synthesis in Sheep: Effect of Temperature, Diet and β-Adrenergic Agonist on Acetyl-CoA Carboxylase and Fatty Acid Synthase

BY

Jacob Adeniyi Moibi

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**

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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **REGULATION OF FATTY ACID SYNTHESIS IN SHEEP: EFFECT OF TEMPERATURE, DIET AND β-ADRENERGIC AGONIST ON ACETYL-CoA CARBOXYLASE AND FATTY ACID SYNTHASE** submitted by **JACOB ADENIYI MOIBI** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL PHYSIOLOGY**.

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DEDICATION

To my wife, **Esther**

my son, **Ayomide**

And

To the glory of God

ABSTRACT

Three separate experiments were conducted to investigate effects of the thermal environment, feeding level, lipid supplementation and β-adrenergic agonist on fatty acid synthesis and lipogenic enzymes in sheep.

In experiment one, acetyl-CoA carboxylase (ACC) activity decreased ($p < 0.05$) by 20 to 33 % in subcutaneous (SC) and mesenteric (MS) adipose tissues during cold exposure and feed restriction. Fatty acid synthase (FAS) activity was reduced ($p < 0.05$) in SC adipose tissue by both cold exposure and feed restriction. Feed restriction also decreased ($p < 0.05$) FAS activity in MS adipose tissue.

In experiment two, the rates of incorporation of [^{14}C]acetate into *longissimus dorsi* (LD) muscle and liver were not influenced by dietary lipid supplementation or environmental temperature. Cold exposure increased ($p < 0.05$) the rates of fatty acid synthesis in perirenal (PR) fat and interacted with lipid supplement to influence fatty acid synthesis in SC and MS fat. Dietary lipid supplementation reduced ($p < 0.05$) the rates of *in vivo* fatty acid synthesis in all three adipose tissues. Environment and dietary lipid did not affect ACC and FAS activity in LD muscle. In liver, ACC activity increased ($p < 0.05$) by 25 % during pair-feeding in the cold, and dietary lipid had the opposite effect (-15 %). In SC and MS adipose tissues, dietary lipid increased ($p < 0.05$) ACC activity by 40%. In PR fat, both cold exposure (+32%) and dietary lipid (+ 38%) increased ($p > 0.05$) ACC activity.

In experiment three, feeding the β-adrenergic agonist, L644,969, increased ($p < 0.05$) the rates of fatty acid synthesis by 38 % in SC and MS adipose tissues in the cold environment. In a warm environment, the β-agonist depressed ($p < 0.05$) the rates of

lipogenesis in SC and PR fat and depressed ACC activity in the three adipose tissues. FAS activity was reduced in PR adipose tissue of agonist treated cold-exposed animals.

Two isoforms of ACC (265 and 280 kDa) were detected in skeletal muscle (*longissimus dorsi*) but only one isoform (265 kDa) was found in the liver and adipose tissue. In *longissimus dorsi* muscle, the 280 kDa was the predominant form, which increased ($p < 0.05$) in response to cold exposure but not to diet. Feed restriction decreased ($p < 0.05$) ACC and FAS protein abundance in SC adipose tissue whereas the cold environment increased ($p < 0.05$) ACC abundance. There was no effect of temperature or feeding level on ACC protein abundance in MS adipose tissue. Dietary lipid reduced ($p < 0.05$) ACC protein abundance in SC adipose tissue. Increased activity and abundance of FAS reflected increases in the rates of *in vivo* fatty acid synthesis in the adipose tissues.

The results indicate that both diet and adrenergic agents can regulate fatty acid biosynthesis in tissues of sheep and that the responses of tissues are modified by environmental temperature and diet.

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List of Abbreviations

α	alpha
μ	micro
ω	omega
β-AR	beta-adrenergic receptor (or adrenoceptor)
ACC	acetyl-CoA carboxylase
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic Adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
Ci	curie
CoA	coenzyme A
ECL	enhanced chemiluminescence
FAS	fatty acid synthase
GE	gross energy (ME metabolizable energy)
GLUT4	glucose transporter 4
h	hour (min minute; s second)
kb	kilobase
kDa	kilodaltons (Da daltons)
MJ	megajoule
MW	molecular weight
NADPH	reduced nicotinamide adenine dinucleotide phosphate
°C	degree Celsius
RNA	ribonucleic acid (mRNA – messenger ribonucleic acid)
rpm	revolutions/minute
SDS - PAGE	sodiun dodecyl sulfate – polyacrylamide gel electrophoresis
V_{max}	maximal velocity
w/v	weight/volume (v/v volume/volume)

1. INTRODUCTION

Long-chain fatty acids synthesized from acetyl-CoA are essential for energy balance, membrane lipid biosynthesis, and other specialized functions in the body. As parts of triacylglycerols, fatty acids represent a major component of stored energy. The rate of *de novo* synthesis of long chain fatty acids from acetyl-CoA is rapid in well-fed animals, especially when the diet has little or no fat, and slow in starved or underfed animals. The enzymatic pathway for the synthesis of fatty acids from acetyl-CoA is present predominantly in adipose tissue of in non-lactating ruminants, and is located within the cytoplasm. Although the molecular and enzymatic regulation of the components for *de novo* fatty acid synthesis are well characterized in some species, the quantitative importance of these components and their physiological functions in ruminants have remained uncertain.

This chapter provides an introduction to several studies conducted to gain insight into the regulation of fat synthesis in ruminant tissues. Attention will focus on key enzymes regulating the pathway of fatty acid synthesis and the influences of nutrition, β-adrenergic agents and thermal environment on the functioning of these enzymes and on the fatty acid biosynthetic pathway.

1.1. FATTY ACID SYNTHESIS IN RUMINANTS

Fatty acid availability in ruminant animals involves both cellular synthesis of fatty acids and uptake of dietary lipid. The majority of *de novo* fatty acid synthesis occurs in the adipose tissue in non-lactating ruminants. In contrast, in non-ruminants, most of the synthesis of fatty acids takes place in the liver. Evidence that adipose tissue is the major

site of fatty acid synthesis in ruminants has been provided by following the fate of [1-^{14}C]acetate in sheep *in vivo* (Mayfield et al. 1966; Ingle et al. 1972b) and by comparing the rates of fatty acid synthesis in homogenates and tissue slices from several ruminant tissues (Hanson and Ballard 1967; Hood et al. 1972; Ingle et al. 1972a). Nevertheless, adipose tissue from ruminants are not homogenous because of differing rates of fatty acid synthesis shown by the different depots (Ingle et al. 1972a,b; May et al. 1994). In *in vitro* measurements, May et al. (1994) showed that the rate of incorporation of [^{14}C]acetate into tissue lipids was higher for subcutaneous adipose tissue compared to intramuscular fat depots. It is likely that the observed differences in fatty acid synthesis rates between adipose tissue sites in the same animal could relate to differences in cell numbers or cell size of the adipose tissue.

1.1.1. Precursors for Fatty Acid Biosynthesis

At present, it is known that acetate is the preferred precursor for fatty acid synthesis in adipose tissue of sheep, cattle and goats and that acetate carbon is incorporated into fatty acids more rapidly than is glucose carbon. Although the basic requirements of synthesis of fatty acids, namely, a supply of acetyl-CoA and of reducing equivalents, are of course the same for both ruminant and nonruminant animals, the way these requirements are met differ quite considerably.

In nonruminants, intramitochondrial metabolism of glucose-derived pyruvate is responsible for the production of acetyl-CoA, which is then translocated into cytosol in the form of citrate from which acetyl-CoA is regenerated by the action of ATP-citrate lyase; the oxaloacetate which is concomitantly produced is converted via malate to

pyruvate. The conversion of malate to pyruvate results in generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) that is required for fatty acid synthesis. The other source of NADPH in nonruminants is derived from the metabolism of glucose by the hexose monophosphate shunt (pentose phosphate pathway). This latter source of NADPH is low in ruminant animals and its place is taken by the isocitrate cycle in which NADPH is generated in the cytosol during the conversion of isocitrate to α-ketoglutarate by NADP-isocitrate dehydrogenase. The activity of NADP-isocitrate dehydrogenase is very high in sheep mammary tissue (Bauman et al. 1970) and the pathway could play a significant role in supplying NADPH for lipogenesis in ruminant mammary tissue. The net result of this pathway is that acetate can furnish a portion of the reducing equivalent needed for fatty acid synthesis.

A similar dependence upon the isocitrate cycle to supply a considerable proportion of NADPH for fatty acid synthesis in ruminant adipose tissue has also been shown by other authors (Ingle et al. 1972a; Yang and Baldwin 1973). Notwithstanding the role of acetate, glucose, as a source of glycerol 3-phosphate, is also required for lipid synthesis as much by ruminants as nonruminants. In this connection the preferential use of acetate for fatty acid synthesis by ruminants and the provision of NADPH by way of the isocitrate cycle can be considered as a teleological way of conservation of glucose. The lack of substantial glucose incorporation into fatty acids in ruminants therefore precludes the citrate cleavage pathway as an important source of reducing equivalents for ruminant tissues. However, under certain conditions such as in fetal and young lambs, glucose can be used for fatty acid synthesis in adipose tissues (Vernon 1979).

Almost all the propionate that reaches the liver of ruminants is metabolized by way of the tricarboxylic acid cycle, following its conversion via methylmalonate to succinate. Very small amounts of propionate can escape hepatic metabolism and thereby can serve as primer units for fatty acid synthesis. Massart-Leën et al. (1983) showed that propionate was utilized for fatty acid synthesis by the mammary glands of lactating goats. Using isolated perfused cow udder, James et al. (1956) also reported that [1-^{14}C]propionate was incorporated into odd-numbered fatty acids isolated from the udder tissue. Moreover, feeding sheep and goats barley-enriched or other high cereal diets can result in an increased proportion of odd-numbered fatty acids along with branched-chain fatty acids in the adipose tissue (Christie 1978; Duncan and Garton 1978).

1.1.2. Overall Regulation of Fatty Acid Synthesis

For fatty acids to meet the complex and varied roles they play in the body, their biosynthesis is regulated at two committed steps by two key enzymes, namely, acetyl coenzyme A carboxylase (Wakil et al. 1983; Hardie et al. 1989) and fatty acid synthase. The reactions catalyzed by the enzymes are a set of complex cytosolic polymerization processes with acetyl-CoA acting as a primer to initiate the process. The overall synthesis of fatty acids is catalyzed by the fatty acid synthase complex, a single polypeptide enzyme, containing seven distinct enzymatic activities.

Multiple mechanisms are known to contribute to the regulation of these enzymes and in a way consistent with the pivotal role of fatty acid synthesis. ACC is regulated in a complex manner both in the short-term, through reversible phosphorylation and allosteric mechanisms (Kim et al. 1989), and in the long-term through modulation of expression of

the ACC and FAS genes. Both hormonal and metabolic signals modulate the activities of the enzymes via allosteric mechanisms involving changes in the polymeric states of the enzyme, and by posttranslational modifications. During lactation, fatty acid synthesis in ruminant adipose tissue is markedly suppressed (Vernon 1988). This may be related, in part, to impairment in an insulin-signalling event. However, adipose tissue from sheep does not respond markedly and reproduceably to insulin in short-term incubations, but it responds clearly during incubations of 24 h or more (Vernon and Finley 1988). With subcutaneous adipose tissue from adult ewes, high rates of fatty acid synthesis can be maintained *in vitro* for at least 6 days in the presence of insulin and dexamethasone (Vernon and Sasaki 1991).

Nutritional responsiveness in the liver as well as in adipose tissue are also critical in the regulation of lipogenic gene expression in animals. The effect of nutrition may involve changes in the cellular concentrations of the enzyme proteins through alterations in gene expression (Hillgartner et al. 1995). In addition to hormonal regulation by insulin (for example), it is now becoming increasingly clear that a group of ligand-activated transcription factors, the peroxisomal proliferator-activated receptors, also play a pivotal role in controlling expression of specific proteins and enzymes involved in lipid metabolism (Smith 1997). Peroxisomal proliferator-activated receptors belong to the nuclear hormone receptor super-family that includes thyroid, steroid, retinoic acid, retinoid and vitamin D receptors. All factors involved in the overall regulation of fatty acid synthesis and metabolic adaptations in ruminants are not completely clear and vary subtly depending on the nutritional and physiological status of the animal.

1.2. REGULATORY ENZYMES OF FATTY ACID SYNTHESIS PATHWAY

1.2.1. Acetyl coenzyme A carboxylase

1.2.1.1. Acetyl-CoA carboxylase reaction and mechanism

Acetyl-CoA carboxylase (ACC) is considered a key regulatory enzyme in the conversion of acetyl-CoA to long chain fatty acids because the concentrations of its substrates and products are far from thermodynamic equilibrium implying that the reaction is catalyzed by a regulatory enzyme and the maximum velocity of the enzyme, as measured in cell extract, is usually the slowest of all the enzymes in the pathway (Goodridge 1991). At least two isoforms of this enzyme have been described in the literature. The rate-limiting nature of ACC has been demonstrated by the specific destruction of ACC-265 mRNA which was carried out by expressing an ACC mRNA-specific ribozyme gene. In that study, the rate of fatty acid synthesis was shown to be commensurate with the amount of ACC mRNA and ACC protein (Ha and Kim 1994).

The overall reaction catalyzed by ACC is a two-step process (Lane et al. 1974) that involves ATP-dependent formation of carboxybiotin followed by transfer of the carboxyl moiety to acetyl-CoA to form malonyl-CoA. The two-step reaction mechanism is shown in reactions 1 and 2 below (Figure 1.1).

HCO_3^- + ATP + E-Biotin → E-Biotin-CO_2^- +ADP + Pi ----------(1)

E-Biotin-CO_2^- + Acetyl-CoA → Malonyl-CoA + E-Biotin ----------(2)

Acetyl-CoA + 7Malonyl-CoA + 14NADPH + 14H^+ + 7ATP → Palmitic acid + 8CoASH + 14$NADP^+$ + 7ADP + 7Pi + 7 CO_2 + 6H_2O ----------(3)

Figure 1.1. Reactions catalyzed by acetyl-coenzyme A carboxylase and fatty acid synthase.

ACC has two distinct catalytic sites, each of which carries out one of the first two reactions shown in Figure 1.1. The first half of biotin carboxylation probably involves initial biotin-independent activation of bicarbonate because ATP/ADP nucleotide is not inhibited by avidin, and a formal carboxy-phosphate intermediate has been observed at least in studies with pyruvate carboxylase (Fry et al. 1985; Phillips et al. 1992). The carboxyl-transferase half-reaction (step 2 above) proceeds via proton extraction which generates a reactive carbanion on the methyl carbon of acetyl-CoA. The product malonyl-CoA is then used for the synthesis of long-chain fatty acids by the multifunctional enzyme, fatty acid synthase. In addition to being an intermediate in the synthesis of long chain fatty acids, malonyl-CoA may also serve as a key regulator in the oxidation of fatty acids, by controlling the activity of carnitine-palmitoyl-CoA transferase 1 (CPT1) at the mitochondrial transport step of long chain fatty acid oxidation (McGarry et al. 1989).

Whereas the highest activity of ACC is found in traditional lipogenic tissues such as in white adipose tissue and mammary gland, ACC activity is also detectable in the heart (Lopaschuk et al. 1994) and in skeletal muscle (Thampy 1989; Bianchi et al. 1990; Winder et al. 1995). Skeletal and heart muscles are relatively nonlipogenic and use long-chain fatty acids as energy sources to support their metabolic activities. Nevertheless, large amounts of ACC have been shown to be present in these tissues (Thampy 1989; Trumble et al. 1991). The ACC in the mitochondria of these tissues is mostly ACC-β (280-kDa) isoform, which has a molecular weight of 275,000 to 280,000 (Bianchi et al. 1990; Saddik et al. 1993; Winz et al. 1994; Trumble et al. 1995).

1.2.1.2. Citrate Activation of Acetyl-CoA Carboxylase

Citrate dependence has been utilized extensively to characterize acetyl-CoA carboxylase. Citrate had been shown to stimulate the incorporation of [^{14}C]acetate into long chain fatty acids in soluble pigeon liver preparations (Brady and Gurin 1952). Watson and Lowenstein (1970) also showed that citrate was an obligatory component of a shuttle system by which mitochondrial acetyl-CoA was transported to the cytosolic compartment, where it became a substrate for acetyl-CoA carboxylase. However, Martin and Vagelos (1962) observed that preincubation of partly purified rat adipocyte acetyl-CoA carboxylase with citrate increased the activity of the enzyme but that citrate itself was not metabolized in their system. Several other lines of evidence have now confirmed that citrate increased the activity of ACC partly purified from a variety of animal tissues (see Volpe and Vagelos (1976), for a review).

Whereas liver ACC was reported to be dependent on citrate for activation (Thampy and Wakil 1985), Thampy and Wakil (1988) found that it is the phosphorylated form of the enzyme that is citrate dependent. Phosphopeptide analysis of ACC-265 isoform has now identified a number of potential phosphorylation sites which are acted upon by a variety of kinases (Hardie 1989; Kim et al. 1989). Phosphorylation of liver ACC by AMP-activated protein kinase and by cAMP-dependent protein kinase has been demonstrated to decrease the V_{max} and increase the $K_{0.5}$ for citrate activation (Kim et al. 1989).

In spite of observations that citrate was an effective activator of acetyl-CoA carboxylase *in vitro*, a role for citrate as a regulator of enzyme activity *in vivo* remained controversial (see Allred and Reilly 1997 for a review), since the concentration of citrate

required for half-maximal activation in tissues was at least three orders of magnitude higher than the concentration of citrate found within the cells. However, one of the undisputed roles of citrate is that acetyl-CoA carboxylase is strongly inhibited by its product, malonyl-CoA, and that citrate competitively overcomes that inhibition. This role of citrate may be important during *in vitro* activity measurements because the isolated enzyme is usually assayed by determining the rate of malonyl-CoA formation as indicated by fixation of $H^{14}CO_3^-$ into its acid-stable form (Thampy and Wakil 1985). Although the need for citrate to overcome malonyl-CoA inhibition during *in vitro* assays of acetyl-CoA carboxylase is apparent, this mechanism may also be important in the regulation of the enzyme *in vivo* under some conditions (Allred and Reilly 1997).

1.2.1.3. Isoforms of ACC and Differential Distribution in Mammalian Tissues

Results from Kim's laboratory (Luo et al. 1989) have shown that the ACC gene is under the control of two promoters designated as promoter I (PI) and promoter II (PII). In these studies they found five different species of mRNA all of which had the same base sequence in the coding region but differed only in their 5'-untranslated region. They classified the mRNAs in the tissues examined as class 1 or class 2 depending on the leading exon, and also suggested that the generation of the different mRNA species was tissue specific and varies according to the physiological condition of the tissue (Lopez-Casillas et al. 1991; Kim and Tae 1994). Acetyl-CoA is also present in isoenzymic forms in different tissues which can be partially ascribed to transcription from two distinct genes (Luo et al. 1989; Ha et al. 1996; Abu-Elheiga et al. 1997).

One isozyme that was first characterized in rat white adipose tissue has a molecular weight of 265,000 Da (Bianchi et al. 1990; Iverson et al. 1990) and will be referred to in this thesis as the ACC-265 or 265-kDa isoform. This isoform is a product of the ACC-α gene which is the principal species in lipogenic tissues. ACC-265 has been purified and characterized from a number of animal tissues, notably from the liver, adipose tissue and mammary gland; sequences of this isoform have been deduced for the enzyme from chicken (Takai et al. 1988b), rat (Lopez-Casillas et al. 1988), sheep (Barber and Travers 1995) and human (Abu-Elheiga et al. 1995).

Based on enzyme isolation, kinetic characterization and selective immunoreactivity with a panel of anti-ACC antibodies, a second rat isoform of 272 to 280,000 Da has also been identified and characterized (Thampy 1989; Louis and Witters 1992; Saddik et al. 1993; Winz et al. 1994; Witters et al. 1994; Trumble et al. 1995). This isoform results from ACC-β which has been recognized as a distinct isoform in heart and skeletal muscles where it is implicated in the regulation of fatty acid β-oxidation (Lopaschuk et al. 1994; Ha et al. 1996; Abu-Elheiga et al. 1997). As yet, the function and role of the multiple isozymes of ACC and diversity of the promoters in mammalian tissues is unclear, but as PI transcript and ACC-α are restricted to principal lipogenic tissues in contrast with the ubiquitous nature of PII transcripts, it is thought that the promoter heterogeneity is related, in part to the tissue specific specialization of the fatty acid storage as opposed to a general requirement to synthesize fatty acids for cell membranes (Travers and Barber 1997).

The discovery of the various isoforms of ACC has emphasized the complexity of regulation of the enzyme in mammalian tissues. This is due to tissue specific expression

of the isozymes (Thampy 1989; Bianchi et al. 1990; Iverson et al. 1990; Louis and Witters 1992). Individual tissues express predominantly one isoform of ACC (Bianchi et al. 1990; Iverson et al. 1990; Winz et al. 1994; Trumble et al. 1995). In rats, ACC-265 is expressed exclusively (Bianchi et al. 1990; Winz et al. 1994) in white adipose tissue. In liver, brown adipose tissue and lactating mammary tissue, the expression of ACC-265 exceeds that of ACC-280 by a factor of 3 to 10 (Bianchi et al. 1990; Iverson et al. 1990). Heart and skeletal muscle predominantly express ACC-280 and this is 10 times that of ACC-265 (Thampy 1989; Bianchi et al. 1990; Iverson et al. 1990). Furthermore, in addition to differences in expression, the isoforms can be coordinately or independently regulated *in vivo* (Bianchi et al. 1990; Witters et al. 1994).

Although both ACC-265 and ACC-280 contain covalently bound biotin as their prosthetic group, ACC-265 is immunologically distinct from ACC-280 (Thampy 1989; Bianchi et al. 1990; Witters et al. 1994). The two isoforms also exhibit different kinetics toward the substrate acetyl-CoA (Witters et al. 1994). Winz et al. (1994) showed that the 265-kDa isoform is phosphorylated by the catalytic subunit of a cAMP-dependent protein kinase at higher rates than is the 280-kDa isoform. Like ACC-265, ACC-280 is a substrate for AMP-activated-protein kinase (AMP-PK) and protein kinase A *in vitro* but direct inhibition of ACC-280 has only been observed in the presence of AMP-activated protein kinase (Winz et al. 1994; Winder et al. 1997), although PKA has now been shown to phosphorylate ACC-280 (Lopaschuk, G. D. personal communication). By comparing the predicted amino acid sequences of ACC-265 with that of ACC-280, Abu-Elheiga et al. (1997) showed that the presence of an additional 142 amino acids at the amino (NH_2) terminal of ACC-280 could account for the differences in molecular weight between the

two isoforms of ACC, and these additional amino acids may be involved in targeting ACC-280 protein toward cellular membranes (mitochondrial, nuclear or endoplasmic).

1.2.1.4. Regulation of Acetyl-CoA Carboxylase in Animal Tissues

Acetyl-CoA carboxylase is under complex dietary and hormonal controls. The enzyme activity may be altered through covalent modification, allosteric control and alteration in its polymeric state (Thampy and Wakil 1985; Hardie 1989). However, specific hormone and intracellular gene regulatory mechanisms that account for the diverse aspects of control of ACC isoform expression are still poorly understood (Brownsey et al. 1997). It is likely that cardinal extracellular signals such as insulin, catecholamines, glucagon and thyroid hormones may play significant roles in regulating ACC expression and function.

In addition to hormones, specific nutrients such as dietary carbohydrates and fat regulate ACC in tissues. Dietary fats rich in polyunsaturated fatty acids are potent inhibitors of hepatic fatty acid and triacylglycerol synthesis (Clarke et al. 1990; Clarke and Abraham 1992), suggesting that long-chain fatty acids or their acyl-CoA derivatives may regulate a key step in the pathway. Their ability to regulate fatty acid biosynthesis may involve regulation of the enzyme activity as well as transcription of the genes which code for the enzyme (Iritani et al. 1995; Cheema and Clandinin 1996). Evidence in the research literature (Clarke et al. 1976; Wilson et al. 1990) indicates that the repression of lipogenic enzymes by dietary fat is due specifically to polyunsaturated fatty acid components in the diet, and that the regulation of the lipogenic enzyme expression, as shown by studies of protein turnover, is reflected in appropriate changes in gene transcription as identified by studies of mRNA expression.

Despite the critical role of ACC in fatty acid synthesis in mammalian tissues, few data on activity of this enzyme have been reported for ruminants (e.g., Ingle et al. 1973; Pothoven and Beitz 1975; Vernon 1976; Scott and Prior 1980; Okine and Arthur 1997). A close relationship between the rate of fatty acid synthesis and activity of ACC in adipose tissue has been found during fasting and refeeding in sheep (Ingle et al. 1973) and fasting in steers (Pothoven and Beitz 1975). Such a correlation was not found in sheep fed various fat supplemented diets (Vernon 1976), during refeeding of fasted steers (Pothoven and Beitz 1975) or in adipocytes from intramuscular fat of steers exhibiting various degrees of marbling (Chakrabarty and Romans 1972). In an attempt to provide a greater understanding of key regulatory processes of fatty acid synthesis, Barber and Travers (1995) have isolated, cloned and characterized multiple ACC transcripts from ovine adipose tissue corresponding to the 5'-untranslated region of ovine ACC cDNA. Comparison of the clones with those of rat and human ACC transcripts in adipose tissues showed significant sequence similarity suggesting that ACC in adipose tissue of sheep may be regulated in a manner similar to its regulation in the adipose tissue of rats and humans.

1.2.2. Fatty Acid Synthase

In animals, the enzymes required for *de novo* biosynthesis of palmitate from acetyl-CoA and malonyl-CoA (reaction 3 of Figure 1.1) are integrated into a single polypeptide chain that, in turn, is encoded by a single-copy gene so that the expression of these enzymes is automatically co-ordinated (Amy et al. 1990). Both FIRE (fatty acid synthase insulin response elements) and ICE (inverted CCAAT element) have been implicated to be

essential in the regulation of FAS promoter (Rangan et al. 1996; Schweizer et al. 1997). The cellular concentration of the multifunctional protein is also regulated in a tissue specific manner by a variety of nutritional, hormonal, physiological and developmental factors (Hillgartner et al. 1995). Thus, in response to starvation, animals may decrease their hepatic fatty acid synthase concentration and restore it after ingestion of a high carbohydrate diet (Volpe and Vagelos 1976; Wakil 1989). A fat-free diet on the other hand will induce synthesis of FAS, and consequently an increase in the synthesis of long chain fatty acids (Wakil et al. 1983; Hillgartner et al. 1995). The synthesis of fatty acids by FAS in this regard is achieved through sequential condensation of two carbon units derived initially from acetyl-CoA.

1.2.2.1. Kinetics of Fatty Acid Synthase Catalyzed Reactions

The synthesis of fatty acids occurs through cyclic elongation resulting in sequential addition of two carbon units to an acyl chain. The kinetics of the overall FAS reaction are quite complex, but under steady state, the kinetics conform to the Michaelis-Menten rate law for the two coenzyme-A ester substrates and NADPH (see Smith 1994 for a review). In the reaction mechanism, the loading of both acetyl and malonyl-CoAs is catalyzed by the same transferase enzyme, each substrate is a competitive inhibitor for the other, and high concentrations of either malonyl-CoA or acetyl-CoA inhibit fatty acid formation (Chang and Hammes 1990). Although FAS appears to be well designed to produce a 16-carbon fatty acid, palmitate is usually not the exclusive product since smaller amounts of myristate (C_{14}) or laurate (C_{12}) may be formed. Fatty acid synthase can also utilize propionyl-CoA in place of the acetyl-CoA primer, therefore generating odd carbon-

number saturated fatty acids mainly the C_{15} and C_{17} (Seyama et al. 1981). This is quantitatively important in ruminants especially when fed a high concentrate diet in which the production of propionate is high in the rumen.

1.2.2.2. Regulation of Fatty Acid Synthase in Animal Tissues

Fatty acid synthase protein abundance in tissues is a function of both its rate of synthesis and rate of degradation. The rate of synthesis can be regulated by controlling the efficiency with which a constant quantity of mRNA is translated into protein or the relative abundance of its mRNA. It has been shown that FAS levels are controlled by the rate of transcription and the stability of its mRNA (Iritani et al. 1992; Hillgartner et al. 1995) and that both nutrition and hormones affect the expression of the FAS gene (Stapleton et al. 1990; Moustaid and Sul 1991; Hillgartner et al. 1995). Dietary regulation of FAS concentration is mediated primarily by controlling transcriptional activity of the gene. Changes in transcription also account for the inhibitory effects of dietary polyunsaturated fatty acids (PUFA) on the synthesis of hepatic FAS in rats (Blake and Clarke 1990; Clarke et al. 1990; Shillabeer et al. 1990).

In suckling rats weaned at 21 d to a balanced diet (50 % carbohydrate, 32 % fat, 18 % protein [% energy]) containing either saturated or polyunsaturated long chain fatty acids, or medium chain fatty acids, it was shown by Foufelle et al. (1992) that the saturated long chain fatty acids and the medium chain fatty acids did not prevent the increases in FAS mRNA concentrations or activities that occurred in the liver. In contrast, the accumulation of FAS mRNA was prevented in the liver of rats weaned to the polyunsaturated long chain fatty acid diet whereas the accumulation of the FAS mRNA

was not affected in adipose tissue. It is likely that the inhibitory effect of PUFA on FAS gene expression was a liver specific phenomenon since PUFA markedly inhibited FAS gene expression in liver but with no effect in adipose tissues (Clarke and Jump 1992; Clarke 1993). This inhibition of gene transcription by dietary fat could lead to a reduction in the abundance of transcript coding for the FAS enzyme. This means that there could be a decrease in tissue content of the proteins involved in lipid synthesis and subsequently a decrease in lipogenic rate (Clarke and Jump 1992).

As in liver, fatty acid synthesis and FAS gene expression in adipose tissue are regulated by hormonal and dietary factors (Mildner and Clarke 1991; Moustaid and Sul 1991). However, it is less clear whether the changes in adipocytes reflect altered expression of gene coding for the lipogenic enzymes. In order to address what factors govern the expression of lipogenic enzymes in adipose tissue, Mildner and Clarke (1991) cloned and sequenced a 1.5-kb cDNA of porcine FAS containing the entire thioesterase domain of the FAS protein and the complete 3'-untranslated region of the transcript. By comparison, the amino acid sequence predicted from the nucleotide sequence of the 1.5-kb FAS cDNA revealed 68 % homology between rat and pig but only 14 % between pig and chicken protein (Mildner and Clarke 1991). By using the 1.5-kb cDNA specific to the porcine FAS it was shown that somatostatin administration reduced FAS mRNA content in adipose tissue by about 80%. Treatment of normal rats with glucagon or dibutyryl cAMP decreased the synthesis of FAS. In starved diabetic rats, insulin was required for the stimulation of FAS synthesis caused by refeeding glucose or a high carbohydrate diet (Paulauskis and Sul 1989). These effects of insulin and glucagon on the synthesis of FAS

were associated with similar changes in the abundance of FAS mRNA *in vivo* (Pry and Porter 1981; Nepokroeff et al. 1984; Paulauskis and Sul 1988).

Furthermore, Soncini et al. (1995) generated transgenic mice carrying a 2.1-kb 5'-flanking promoter region of the FAS gene fused to a chloramphenicol acetyltransferase (CAT) reporter gene. They showed that the CAT activity was increased in both liver and white adipose tissue when fasted animals were refed a high carbohydrate fat free diet, but was not detectable in other tissues such as kidney, muscle, lung and heart. These changes in CAT activity and CAT mRNA occurred in parallel to changes in endogenous FAS mRNA levels. Thus, FAS gene transcription occurs in lipogenic tissues only and the 2.1-kb 5'-flanking region of the rat FAS gene was sufficient for the induction of the FAS gene by fasting / refeeding. Increased insulin and decreased glucagon brought about by the nutritional manipulation of fasting / refeeding may also contribute to the changes in the FAS gene expression.

1.3. INFLUENCE OF THERMAL ENVIRONMENT ON THE REGULATION OF FATTY ACID SYNTHESIS

One of the major agents stimulating lipid mobilization is the catecholamine group. Mobilization of stored fat appears to play a role in adaptation of mammals to a number of different situations such as exposure to a cold environment and starvation. During cold exposure, ruminants must increase their metabolic rates in order to maintain normal core body temperature. Along with several other changes that take place within the body, plasma concentration of free fatty acids (FFA) quickly increase after the onset of a cold stress in sheep and cattle and the levels remain elevated during more prolonged exposure

to cold (Young 1975; Sano et al. 1995). Also, there is a significant increase in blood flow to adipose tissue in cold-exposed sheep (McBride and Christopherson 1984) which would assist FFA release by increasing the rate of presentation of albumin binding sites of FFA.

The regulation of fatty acid synthesis in ruminants during chronic cold-exposure has not been specifically studied. However, there is good evidence to indicate that during cold exposure, the mobilization of long-chain fatty acids from adipose tissue will be controlled primarily by the sympathetic nervous system. Christopherson et al. (1978) have shown that sympathetic nervous activity remains elevated during cold exposure of sheep and this could promote fat mobilization. Moreover, the primary factor in the regulation of fatty acid synthesis during cold exposure is likely to be the overall energy balance of the animal as would be mediated by the net effect of changes in secretion of a number of anabolic and catabolic hormones which affect the balance between lipid synthesis and lipid mobilization.

In animals maintained on a low fat diet, increased heat production is associated with high rates of fatty acid synthesis, in both short-term (Rath et al. 1979) and in long-term cold exposure (Trayhurn 1979). Trayhurn (1981) showed that the rate of fatty acid synthesis was more than three times higher in white adipose tissue of cold-acclimated mice compared to the warm-acclimated group. In rat brown adipose tissue McCormack and Denton (1977) showed that both the rates of fatty acid synthesis and the activity of acetyl-CoA carboxylase were increased in response to cold adaptation. Buckley and Rath (1987) showed that fatty acid synthesis decreased in rat adipose tissue in response to acute cold-exposure but the activity of acetyl-CoA carboxylase was increased under the same condition. In contrast, normal increases in acetyl-CoA carboxylase activity were

abolished by intraperitoneal injection of noradrenaline into rats exposed to a thermoneutral environment (Gibbins et al. 1985).

Lefaucheur et al. (1991) showed increases in the activities of NADPH-supplying enzymes (malic enzyme and glucose-6-phosphate dehydrogenase) in subcutaneous adipose tissue of cold-exposed pigs. The increase in ACC activity in adipose tissue of the cold-exposed pigs was not significant, so no clear conclusion of the effect of temperature on adipose tissue lipogenesis could be made.

In conditions of equal rates of gain, Verstegen et al. (1985) showed that pigs housed at a low environmental temperature exhibited a reduction in lean:fat ratio in body weight gain, suggesting that the animals were depositing fat in the cold environment. Similarly, Lefaucheur et al. (1991) showed that pigs maintained at a cold temperature of 12 °C (lower than the lower critical temperature of pigs) from 8 kg live weight to slaughter at about 92 kg live weight also had more dissectable fat in the hams than those maintained at +28 °C. They also showed that, the increase in the ham subcutaneous adipose fat, together with a decrease in leaf fat in that experiment suggested a small shift in fat deposition from internal to external sites in pigs maintained in the cold. This suggests that the peripheral adipose tissue was relatively more active in fat synthesis and deposition during cold temperature exposure.

In an attempt to determine the response of rat brown and white adipose tissue to environmental or nutritional stress, Klain and Hannon (1977) showed an increase in the size of brown adipose tissue in cold-exposed compared to the control warm-exposed rats. In contrast, however, cold exposure completely abolished fatty acid synthesis and led to a slight loss of total lipids from white adipose tissue.

1.4. NUTRITIONAL AND HORMONAL REGULATION OF FATTY ACID SYNTHESIS AND LIPOGENIC ENZYMES IN ANIMALS

Low temperature exposure may adversely affect the efficiency of livestock production in areas where mean daily temperatures fall below the freezing point for a substantial part of the year. In experiments with shorn lambs, low temperatures have been found to increase feed intake (Ames and Brink 1977; Ekpe 1998) and maintenance requirements (Graham et al. 1959) and to decrease the digestibility of feed. Feeds with a high-energy density such as concentrate or those with a high-fat content offer a means of increasing energy intake without increasing total dry matter intake. Although fat supplements are commonly fed in the beef feedlot industry, the effects on feed conversion, daily gain and carcass characteristics are variable (Brandt and Anderson 1990; Bock et al. 1991; Huffman et al. 1992; Petit et al. 1994) but can be used to alter the composition of adipose tissues provided that the fat source is protected from rumen biohydrogenation.

Dietary carbohydrate and fats have been shown in several studies to regulate lipogenic enzymes. Both *in vivo* and *in vitro* studies with monogastric species strongly support the conjecture that marked increases in the levels of mRNA coding for lipogenic enzymes and in the activities of lipogenic enzymes that occur in liver and adipose tissue in response to a high carbohydrate diet are dependent on an increase in plasma glucose and insulin concentrations (see Towle et al. (1997) for a review). Optimal *in vivo* induction of lipogenic enzymes by dietary carbohydrate also requires triiodothyronine (Mariash et al. 1980). While glucose can stimulate ACC and FAS in the absence of insulin, the addition of insulin strongly potentiates its effects. This is likely due to effects

of insulin on GLUT4 transporter of the adipocytes, which is recruited from intracellular sites to the plasma membrane in the presence of insulin.

In liver, the activity of ACC is regulated by alterations in nutritional status of the animal. Short-term changes in enzyme activity are mediated by allosteric and covalent modification mechanisms (Cohen and Hardie 1991). ACC is also controlled by long-term alteration in enzyme concentrations. For example, the concentration of ACC in liver from starved chickens and rats is low. Feeding a high carbohydrate, low fat diet stimulates a 10-fold increase in the amount of ACC (Takai et al. 1988a). These diet induced changes in ACC concentration are accompanied by comparable alterations in the synthesis rate and mRNA abundance for ACC, suggesting that regulation occurs at a pretranslational step (Takai et al. 1988a; Hillgartner et al. 1996). The general pattern of ACC activity and mRNA in response to dietary manipulations is likely similar across species.

In sheep, Okine and Arthur (1997) showed that feeding a high concentrate barley-based diet stimulated both ACC and FAS activity in subcutaneous adipose tissue compared to a roughage control. In nonruminants, FAS is affected by nutritional status of the animal. The activity is reduced during periods of fasting and returns to normal during refeeding. Ingle et al. (1973) observed a decrease in FAS activity after a 48-h fast in sheep; this decline in activity continued during a 192-h fast. In this same study, a correlation was high between *in vitro* lipogenic capacity and ACC activity during fasting and refeeding. However, Scott and Prior (1980) did not find any consistent response in ACC to dietary energy density in steers.

Cold acclimation has been shown to stimulate both the secretion and peripheral utilization of thyroid hormones in animals allowed to consume a high-energy diet

(Tomasi and Horwitz 1987; Dauncey 1990). The effects of long-term acclimation to a low temperature on thyroid hormone are controversial, they may be increased (Herpin et al. 1987), decreased (Tomasi and Horwitz 1987) or unchanged (Lefaucheur et al. 1991). The discrepancy between results could be due to the fact that only total T_3 and T_4 concentrations are generally measured, although the active hormone is T_3 and the biological effect is mediated by the free form. The total levels of T_3 may decline but effective free hormone may increase if thyroxine-binding globin levels decreases simultaneously. On balance, both secretion and peripheral utilization of thyroid hormone seems to be increased in a cold environment.

Thyroid hormone has been reported to stimulate the expression of ACC and FAS mRNA in liver and adipose tissues. Blennemann et al. (1992) examined the regulation of fatty acid synthesis by thyroid state in a wide range of rat tissues. They showed that fatty acid synthesis in liver was extremely responsive to thyroid status. Blennemann et al. (1995) also showed that both ACC and FAS mRNA levels were increased in rat epididymal and retroperitoneal fat during a hyperthyroid compared to a euthyroid state. Hypothyroidism also decreased ACC and FAS mRNA in these fat depots when compared to the euthyroid state. In chicken liver, Kameda (1995) showed that FAS activity in methimazol-treated hypothyroid chickens was 48 % of the activity in the euthyroid animals. In the hypothyroid animals, the author showed that T_3 (200 μg / 100 g body weight) increased FAS activity by 144 % whereas T_3 showed no significant effect on the enzyme activity in euthyroid animals. On the other hand, T_3 reduced the level of mRNA for FAS in both the euthyroid and the hypothyroid chicken, probably due to decreased rate of transcription. Data on thyroid hormone regulation of lipogenic activity in white

adipose tissue are variable. While there are reports that tritium incorporation into fatty acids was highest in euthyroid white adipose tissue, with lower levels being recorded in both hypo- and hyperthyroid animals (Blennemann et al. 1992), others have reported stimulatory effects of thyroid hormone (Gnoni et al. 1980; Freake et al. 1989).

The results of Blennemann et al. (1995) were the first published information on the role of T_3 in the regulation of ACC and FAS mRNA in white adipose tissue. These authors contended that if T_3 uniformly stimulated the expression of both ACC and FAS mRNAs in white adipose tissue it must have other effects which interrupt the expected increase in fatty acid synthesis. The most likely explanation for this lies with ACC, which unlike FAS is also regulated at the level of enzyme activity. It is known that hyperthyroidism sensitizes white adipose tissue to the effects of circulating catecholamines (Van Inwegen et al. 1975; Rapiejko et al. 1989). This may lead to enhanced intracellular levels of cAMP and fatty acyl CoA, which both inhibit ACC activity (Volpe and Vagelos 1976). Thus, although the nuclear actions of T_3 on lipogenic genes are directionally similar in white adipose tissue and liver, secondary effects of the hormone may result in quite different effects on fatty acid synthesis (Blennemann et al. 1995).

1.5. ADRENERGIC REGULATION OF LIPOGENIC ENZYMES

Adrenergic receptors mediate the physiological actions of the hormones adrenaline and noradrenaline. Since the original classification of adrenoceptors by Ahlquist (1948) into alpha and beta types, several members (or subtypes) of these classes have been characterized. Many have been implicated in the control of various metabolic processes

by catecholamines. The mechanisms of the effects of β-adrenoceptor (β-AR) agonists are probably due to the coupling of Gs (guanine nucleotide – stimulatory) protein, leading to activation of membrane bound adenylyl cyclase to produce cAMP (Ruffolo 1994).

Cunningham (1965) presented evidence to indicate the possibility of changing mammalian growth by administration of agents (such as nicotine, caffeine and epinephrine) that directly and indirectly might function by changing intracellular concentration of cAMP. Since then, several published data have indicated the growth promoting or repartitioning effect of β-AR agonists. Two norepinephrine analogs clenbuterol and cimaterol have been shown to yield increased carcass muscle mass when fed to sheep (Beermann et al. 1987) and cattle (Ricks et al. 1984). Nash et al. (1994) also showed that chronic administration of β-AR agonist cimaterol improved nitrogen retention in sheep and produced a substantial and prolonged improvement in protein gain.

β-Adrenoceptor agonists exert their effects on livestock growth at least, in part, by directing lipolysis and lipogenesis in adipose tissue. Mersmann (1984a,b), and Rule et al. (1987) showed that isoproterenol, a β-AR agonist, stimulated lipolysis by porcine adipose tissue *in vitro*. Peterla and Scanes (1990) also showed that in the absence of insulin, isoproterenol significantly increased the release of free fatty acids by porcine adipose tissue *in vitro*. Cimaterol also stimulated FFA release but depressed the rates of lipogenesis in that study. In contrast, these authors showed that clenbuterol in the absence of insulin did not significantly influence FFA release but depressed lipogenic rates by 64 %. Several β-AR agonists also acutely elevate plasma FFA in cattle (Blum and Flueckiger 1988; Eisemann et al. 1988) but the response was suppressed with chronic

administration of the β-AR agonist in cattle (Eisemann et al. 1988) and in sheep (Beermann et al. 1987).

Whereas the general contention has been that catecholamines inhibit fatty acid synthesis in adipose tissue through mechanism involving β-AR mediated inactivation of ACC (Denton and Halperin 1968), Wilson (1989) showed that the β-AR agonist BRL 26830 increased the rate of fatty acid synthesis in white adipose tissue from rats by 135 % during the 30 to 60 min period after oral dosing (5 mg kg^{-1}). The same compound produced a 78 % decrease in the rate of fatty acid synthesis in brown adipose tissue of the same animals over the same period of time with a corresponding decrease (-75 %) in activity of ACC. However, no data were presented on the effect of the β-AR agonist on ACC activity in white adipose tissue.

In sheep chronically fed a β-adrenergic agent (2 ppm clenbuterol) for 40 to 44 d, Coleman et al. (1988) showed that the rate of incorporation of [^{14}C]acetate into fatty acids was not significantly different from the control untreated animals. This lack of effect of clenbuterol was contrary to decreased lipogenic rates *in vitro* in subcutaneous adipose tissue from clenbuterol fed sheep (Hu et al. 1988), and in epididymal adipose tissue of clenbuterol-fed rats (Duquette and Muir 1985). The fact that both increased and decreased rates of lipogenesis have been reported in several species following β-AR agonist feeding may suggest that the effect of β-AR agonist on fat metabolism was elicited through different mechanisms in these species. Alternatively, several effects of β-AR measured *in vitro* may not truly reflect the mode of action of these compounds in reducing fat accretion *in vivo*.

1.6. SUMMARY

In the preceding sections of this chapter, the thermal environment to which an animal is exposed was demonstrated to have an influence on the regulation of fatty acid synthesis. This may occur through alterations in the concentrations of hormones such as catecholamines (through α- or β-AR mechanisms), or changes in metabolite levels (e.g., ATP: ADP ratio) by modulating the energy requirement of the animal. In normal fully energized cells (as in fully fed animals), the ATP: ADP ratio is maintained at a high level by oxidative phosphorylation. However, if the animal experiences some stress (e.g. cold stress or feed restriction), this interferes with ATP production and lowers the ATP: ADP ratio (Hardie et al. 1994). It has been shown that several stressors that deplete ATP activate AMP-activated protein kinase leading to inactivation of ACC and a marked inhibition of fatty acid synthesis in isolated rat hepatocytes (Moore et al. 1991; Corton et al. 1994; 1995).

Also indicated was that dietary manipulation, including fasting and refeeding, diet type and diet composition such as the amounts of carbohydrate or fat in the diet, affected significantly both the lipogenic enzyme properties and the rates of fatty acid synthesis in tissues of animals. Thirdly, as a way of manipulating carcass fat depot and the rates of fatty acid synthesis in animals, several adrenergic agents have been employed with the result leading to an increase or a decrease or in some cases no change in the observed rates of fatty acid synthesis.

This background information has provided evidence that dietary level, type and composition play important roles in regulating fatty acid synthesis both in ruminant and nonruminants. However unlike other species in which the effect of environmental

temperature exposure (Trayhurn 1981; Buckley and Rath 1987; Lefaucheur et al. 1991) has been investigated, there is little or no information concerning the effect of thermal environment on its own or in association with either diet or beta-adrenergic agonist on the rates of fatty acid synthesis and lipogenic enzyme characteristics in sheep.

Moreover, a major problem facing the livestock industry is the production of carcasses containing excess fat. Research needs to be geared at seeking ways to alter the partitioning of nutrients away from adipose tissue deposition and towards muscle accretion. Because adipose tissue contributes to energy reserves in the animal and a greater quantity of energy is required to produce one kg of adipose tissue than is required to produce one kg of muscle, successful repartitioning should result in an improvement in animal efficiency. Understanding the effects of thermal and nutritional environments on regulation of regional fat deposition could have major implications for energetic efficiency of the animal and the potential quality of meat products. Thorough understanding of the processes of fat deposition in ruminants may contribute substantially to quality indices and improved costs management.

1.7. HYPOTHESES

A series of experiments was designed to test the following hypotheses.

1. Thermal environmental exposure affects the rates of fatty acid biosynthesis in tissues from lambs.
2. The effects of thermal environment on fatty acid synthesis are the results of alterations in the activity and protein abundance of the key lipogenic enzymes ACC and FAS.

3. The effects of thermal environment on rates of fatty acid biosynthesis and lipogenic enzymes are modulated by plane of nutrition.
4. Thermal exposure and dietary fat alter metabolic properties of the animal, which may then alter the rates of fat synthesis.
5. The effects of β-AR agents on fat accretion and nutrient partitioning are mediated through their effects on rates of fatty acid synthesis and on lipogenic enzymes, and that cold temperature exposure alters the responses of animals to beta-adrenergic agonists.

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2. Acetyl-CoA Carboxylase and Fatty Acid Synthase Activities in Adipose Tissue of Ruminants: Effect of Cold Exposure and Dietary Feeding Level

2.1. INTRODUCTION

Control of acetyl-CoA carboxylase (EC 6.4.1.2; ACC) and fatty acid synthase (EC 2.3.1.85; FAS) in adipose tissue of ruminants is of special interest because the products of their metabolic pathway play an important role in energy storage and may also be mobilized for oxidation to meet dietary energy deficit or for increased thermogenic requirements. The regulatory mechanisms for ACC, a rate limiting enzyme in the biogenesis of long chain fatty acids, and FAS, which may also be rate limiting in long-term synthesis of fatty acids, are extremely complex at both the gene level (Clarke et al. 1990; Luo and Kim 1990,) and at the protein level (Hardie 1989; Kim et al. 1989). Acetyl-CoA carboxylase and FAS are known to be regulated by alterations in hormonal and nutritional status (Wakil et al. 1983; Kim and Freake 1993, 1996; Hillgartner et al. 1995). While short-term changes in ACC activity are mediated by allosteric and/or covalent modification mechanisms (Kim et al. 1989; Cohen and Hardie 1991; Hillgartner et al. 1995), changes in FAS activity and long-term changes in ACC activity are generally controlled by alterations in enzyme concentrations (Hillgartner et al. 1995). Whereas abundant literature exists with respect to ACC and FAS regulation in tissues from non-ruminant animals there is little information with respect to tissues from ruminant species.

When ruminants are exposed to a cold environmental temperature there is a stimulation of metabolic activity and an increase in noradrenaline release and turnover (Graham and Christopherson 1981). Catecholamines (adrenaline and noradrenaline) are

known to modulate lipid metabolism in white and brown adipocytes (Young et al. 1982; Vallerand et al. 1983). As fatty acid synthesis in ruminants occurs mainly in adipose tissue, the response to cold exposure mediated by sympathetic activation in this tissue may be important in the regulation of fatty acid synthesis by regulating the activity of ACC and FAS.

Previous studies have demonstrated changes in lipogenic enzyme properties in response to dietary manipulations such as fasting or starvation and refeeding (Moir and Zammit 1990; Hillgartner et al. 1995; Winder et al. 1995). In adipose tissue, hormonal changes that occur following dietary restriction and cold exposure have the potential to inhibit both ACC and FAS since many of these hormones regulate tissue concentration of the enzyme protein. Hormones such as adrenaline, the levels of which may increase during stress and when animals are exposed to a cold environment, may affect ACC and FAS by increasing the level of intracellular cAMP concentration in adipose tissue. The effect of a high cAMP concentration may be mediated by rapid phosphorylation of ACC by an AMP-activated protein kinase (Hardie et al. 1989; Davies et al. 1990). This may lead to a rapid inhibition of ACC enzymatic activity, thereby providing a central cascade for the regulation of different aspects of lipid metabolism. This type of covalent regulation has not been reported for FAS but its regulation may involve changes in enzyme concentration, which are controlled by the rate of transcription and (or) the stability of its mRNA.

In view of the importance of the fatty acid synthesis pathway to fat deposition and carcass quality attributes in animals, to date there has been no published information on the potential effects of environmental temperature and feed restriction on regulation of

enzymes of the fatty acid biosynthetic pathway in adipose tissue from ruminant animals. The objective of this study was to investigate the effects of exposure to a cold environment on the activity of two key regulatory enzymes of fatty acid synthesis in peripheral and internal adipose tissue in sheep fed two levels of feed intake.

2.2. MATERIALS AND METHODS

2.2.1. Materials

[^{14}C]bicarbonate was purchased from Dupont NEN (Boston, MA. U.S.A.). Bovine serum albumin (essentially fatty acid free), aprotinin, leupeptin, pepstatin A, soybean trypsin inhibitor, phenylmethanesulphonyl fluoride (PMSF), polyethylene glycol (PEG) 8000, acetyl-CoA, malonyl-CoA and nicotinamide adenine dinucleotide phosphate (reduced form; NADPH) were purchased from Sigma Chemical Co. (St. Louis, MO. U.S.A.). EcoLite™ liquid scintillation fluor was from ICN (Costa Mesa, CA. U.S.A.). Enhanced chemiluminescence (ECL) western blotting reagents and Hyperfilm-ECL were purchased from Amersham Life Science (Buckinghamshire, England). Nitrocellulose transfer membranes were purchased from Micron Separation Inc. (Westborough, MA. U.S.A.). Peroxidase-conjugated-streptavidin was purchased from Jackson ImmunoResearch Laboratories, Inc. (WestGrove, PA. U.S.A.). Mouse anti-fatty acid synthase monoclonal antibody was purchased from Chemicon International Inc. (Temecula, CA. U.S.A.)

2.2.2. Animals and Management

Twenty-four Suffolk-cross 6 mo old wether lambs were allocated to temperature (cold, 0 ± 2 °C or warm, 23 ± 2 °C) and feeding (*ad libitum* or restricted) treatments in a 2 X 2

factorial arrangement in a completely randomized design. The four treatments were cold-*ad libitum* (CA), cold-restricted (CR), warm-*ad libitum* (WA) and warm-restricted (WR). All animals were cared for according to guidelines of the Canadian Council on Animal Care (1993). Animals were housed in individual pens (180 x 90 x 90 cm) and were fed on alfalfa pellet diet (9.08 MJ estimated ME kg^{-1} DM, 17% CP). Restricted-fed animals were given 1.35 and 1.75 x maintenance requirement of the thermoneutral zone, respectively, for warm and cold environments. Daily feed allotment for restricted groups was adjusted weekly according to body weights based on published information on heat production and total body insulation of shorn sheep (Christopherson and Young 1981; McBride and Christopherson 1984 a,b; NRC 1985). Water and cobalt-iodized salt blocks were continuously available to all the animals. Lambs were adapted to treatments for 28 d before the experiment and the study was divided into three periods of five weeks each. During period two, warm-restricted (WR) and cold-restricted (CR) groups were removed from the experiment and the animals were fed *ad libitum* in a thermoneutral environment to prevent declines in body condition prior to period three. All treatments and metabolic measurements were as described by Ekpe (1998). At the end of 15 wk, the wethers were killed using captive bolt stunning, followed by rapid exsanguination. Subcutaneous (peripheral) and mesenteric (internal) adipose tissues were rapidly sampled within 10 min of killing and snap frozen in liquid nitrogen. Frozen tissues were stored at –80 °C prior to measurement of ACC and FAS activities and western-blot analyses.

2.2.3. Acetyl-CoA carboxylase Assay

Frozen tissue was pulverized under liquid nitrogen and approximately 1 g was

homogenized with a buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, (pH 7.5 at 4 °C), 50 mM NaF, 0.25 M mannitol, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 5 mM sodium pyrophosphate, 1 mM PMSF, 1 mM benzamidine and 4 µg/ml soybean trypsin inhibitor as described previously (Lopaschuk et al. 1994; Makinde et al. 1997). The buffer also contained 4 µg/ml each of aprotinin, leupeptin, and pepstatin A.

Homogenates were centrifuged at 14,000 x g for 20 min at 4 °C. The supernatant samples were made 2 % PEG, stirred for 10 min at 4 °C and then centrifuged at 10,000 x g for 10 min using a JA-21 rotor (Beckman Instruments Inc., Irvine, CA). Acetyl-CoA carboxylase protein was precipitated from the supernate in 10 % PEG 8000 solution, stirred on ice for 10 min and centrifuged as before. The precipitate was collected and washed with 10 % PEG 8000/homogenizing buffer. After centrifugation (10,000 x g, 10 min) the pellet was resuspended in a resuspension buffer containing 100 mM Tris-HCl (pH 7.5 at 4 °C), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM Na pyrophosphate, 10 % (w/v) glycerol, 0.02 % sodium azide, 4 µg/ml soybean trypsin inhibitor and 1 mM benzamidine. The protein content of the resuspended enzyme was measured using Bradford's method (Bradford 1976).

Acetyl-CoA carboxylase activity was determined in triplicate using assay procedure described by Lopaschuk et al. (1994) except that the HPLC step was replaced by measuring the rate of incorporation of [^{14}C]bicarbonate into acid-stable compounds (malonyl-CoA) (Thampy and Wakil 1985). Final concentrations of assay components were 60.6 mM Tris-acetate, 2.12 mM ATP, 1.32 µM β-mercaptoethanol, 5.0 mM Mg

acetate, 10 mM potassium citrate, 1.06 mM acetyl-CoA, 18.18 mM $NaHCO_3$, 0.33μCi/μmol $NaH^{14}CO_3$ and 1 mg/ml fatty acid free-BSA (pH 7.5). The reactions were started by addition of 10 μl of enzyme preparation (preincubated for 5 min) in a final assay mixture of 165 μl. After a 4 min incubation at 37 °C (in a shaking water bath) the reaction was stopped by addition of 25 μl of 10% perchloric acid. Reaction tubes were immediately placed in a dessicator under vacuum to remove unreacted label as $^{14}CO_2$ and tubes were centrifuged at 3500 rpm (2900 x g) for 20 min with a Beckman J-6M/E centrifuge and bucket rotor JS-5.2 (Beckman Instruments Inc., Irvine, CA). After centrifugation 160 μl of the supernate was transferred into a glass scintillation minivial and evaporated to dryness at 80 °C under gentle vacuum. The residue was dissolved in 100 μl of H_20 and mixed with 4 ml scintillation fluid for determination of radioactivity.

An initial preliminary experiment indicated that the reaction and enzyme concentration employed for the assays were optimal for the determination. The assay procedure described above also utilized 10 mM potassium citrate because citrate had earlier been shown in preliminary studies (see Figure 2.1) to be required for maximal activity. Acetyl-CoA carboxylase activity was expressed as nmol of ^{14}C-bicarbonate incorporated into malonyl-CoA.min^{-1}.mg $protein^{-1}$.

2.2.4. Fatty acid synthase assay

Frozen adipose tissue samples were pulverized in liquid nitrogen and approximately 2 g was homogenized (30 s at 4 °C) in three volumes of phosphate bicarbonate buffer (70 mM $KHCO_3$, 85 mM K_2HPO_4, 9 mM KH_2PO_4, 1 mM DTT, pH 8) using a teflon Potter-Elvehjem homogenizer. The homogenate was centrifuged (model J2-21) in a JA-20 rotor

type (Beckman Instruments Inc., Irvine, CA) at 10,000 x g for 10 min and the supernatant fluid was centrifuged (105,000 x g for 60 min at 4 °C) using a Solvall® (Ultra *pro*80™, Du Pont Co. DE, USA) centrifuge to obtain adipose tissue cytosol. The retained supernatant was brought to saturation with saturated ammonium sulfate solution (containing 3 mM EDTA and 1 mM β-mercaptoethanol) and stirred for 60 min on ice. The precipitate was collected by centrifugation at 105,000 x g for 60 min. The pellet was dissolved in 5% of original volume of the homogenate buffer and was centrifuged briefly in a microcentrifuge to remove insoluble protein. The protein content of the supernatant was assayed according to Bradford (1976).

Fatty acid synthase activity was determined in duplicate according to the method of Nepokroeff et al. (1975) by measuring the malonyl-CoA and acetyl-CoA dependent oxidation of NADPH. The reaction was carried out at 30 °C using a Varian Cary UV-visible automated spectrophotometer equipped with a temperature controller (Varian Australia Pty Ltd. Mulgrave, Victoria, Australia). For each assay, reference (blank) and sample cuvettes were measured simultaneously and the decrease in absorbance at 340 nm was recorded by a computer-software-integrator attached to the spectrophotometer. The reaction was linear in the ranges employed. Final concentrations of assay mixture were (per ml) potassium phosphate buffer 500 µmol, EDTA 1µmol, β-mercaptoethanol 1 µmol, acetyl-CoA 35 nmol, malonyl-CoA 100 nmol and NADPH 100 nmol. The concentration of NADPH oxidized was calculated using the following equation:

$$\Delta C = \Delta A/E$$

Where ΔC = change in concentration of NADPH, ΔA = change in absorbance and E = extinction coefficient of NADPH ($E_{340\,nm} = 6.22\ mM^{-1}\ cm^{-1}$).

Fatty acid synthase activity was expressed as nmol NADPH oxidized.min^{-1} $mg\ protein^{-1}$.

2.2.5. Western Blot Analyses of ACC and FAS

Twenty µg of subcutaneous or 25 µg of mesenteric adipose tissue protein was prepared in a sample buffer containing 10 % glycerol, 62.5 mM Tris, pH 6.8, 2 % β-mercaptoethanol and 0.025 % bromophenol blue. After being heated in boiling water for 5 min, the samples were subjected to sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (1970). For each tissue, two identical sets of gels were run simultaneously in the presence of 0.1 % SDS, 25 mM Tris, 192 mM glycine, pH 8.3, at 100 volts for 2.5 h. After electrophoresis on a 5 % gel (Appendix 2), the fractionated proteins were transferred to nitrocellulose membranes. For ACC, one set of membranes was probed with peroxidase-labelled-streptavidin. For FAS, a second set of membranes was probed with a primary mouse anti-fatty acid synthase monoclonal antibody and a secondary goat anti-mouse-horse-radish-peroxidase. Chemiluminescent detection was performed on the membranes using an enhanced chemiluminescence western blotting kit, rapidly followed by autoradiography with HyperFilm-ECL.

2.2.6. Statistical Analyses

Two-way analysis of variance was used to determine significant differences in means among the various treatment groups using the GLM procedure (SAS 1995). The model included temperature, feed and temperature x feed effects. Each item in the model was tested against the residual error. After an overall significant treatment effect was established the pdiff procedure of GLM was used to evaluate differences in various

comparisons. Students t-test was used to compare if there were any differences in enzyme activity between depot sites within any given treatment. The level of significance for each test was set at $p < 0.05$.

2.3. RESULTS

2.3.1. Acetyl-CoA carboxylase activity

Table 2.1 shows ACC activity in subcutaneous and mesenteric adipose tissues from sheep following their exposure to different environmental conditions and feeding levels. Acetyl-CoA carboxylase total activity measured in cytosolic extracts on the resuspended PEG precipitate was lowered ($p < 0.05$) by 20 to 33 % in subcutaneous and mesenteric adipose tissues by cold exposure when compared to tissues from animals in the warm group. In both adipose tissue depots, feeding level effects were significant ($p < 0.05$) with feed restriction reducing ACC activity by 23 to 32 %. There was no significant temperature x feed interaction for ACC activity so results are presented for main treatment effects only. Means (± SE) for the individual treatment groups were WA 3.81 ± 0.564; WR 2.47 ± 0.191; CA 2.53 ± 0.256 and CR 1.88 ± 0.188 nmol $H^{14}CO_3.min^{-1}.mg\ protein^{-1}$ for subcutaneous adipose tissue, and, WA 3.58 ± 0.475; WR 2.83 ± 0.149; CA 2.79 ± 0.193; and CR 2.08 ± 0.140 nmol $H^{14}CO_3.min^{-1}.mg\ protein^{-1}$ for mesenteric adipose tissue. Acetyl-CoA carboxylase activities within each treatment were compared by paired t-test and were similar ($p > 0.05$) in the two fat depots (Table 2.1).

2.3.2. Fatty acid synthase activity

Table 2.2 shows that there were marked differences in FAS activity depending upon

treatment group as well as the specific fat depot. Both cold exposure and feed restriction reduced ($p < 0.05$) FAS activity in subcutaneous adipose tissue. Feed restriction resulted in a significant decrease ($p < 0.05$) in FAS activity in mesenteric adipose tissue whereas temperature had no effect. There was a tendency for a temperature by feeding level interaction ($p = 0.08$) for both tissues. The mean FAS activity for individual treatments were WA 3.02 ± 0.389; WR 1.67 ± 0.192; CA 1.69 ± 0.189 and CR 1.26 ± 0.166 nmol NADPH.min^{-1}.mg protein^{-1} for subcutaneous adipose tissue, and WA 6.70 ± 0.721; WR 2.94 ± 0.562; CA 4.79 ± 1.00 and CR 4.10 ± 1.00 nmol NADPH.min^{-1}.mg protein^{-1} for mesenteric adipose tissue.

A paired t-test analysis of the data presented in Table 2.2 showed that adipose tissue samples from the two anatomical locations (peripheral and internal) from animals on the same treatment differed in enzyme activity. The activities of FAS in the peripheral subcutaneous fat were lower ($p < 0.05$) when compared to those of the internal mesenteric depot.

2.3.3. SDS/PAGE and Western-Blot Analyses

Western blot analyses of acetyl-CoA carboxylase demonstrated that only one isoform (the 265-kDa isoform) was present in both subcutaneous and mesenteric adipose tissues. Results of quantification of ACC-265 in cellular cytosolic extracts, to some extent, reflected differences in the enzyme activity studies. Figure 2.2 shows the levels of ACC protein expression in both subcutaneous and mesenteric adipose tissues. In subcutaneous adipose tissue, ACC protein expression was depressed ($p < 0.05$) by feed restriction whereas cold exposure of the lambs had the opposite effect ($p < 0.05$). There was also a

trend for a temperature x feeding level interaction ($p = 0.06$). The means for the individual treatments in SC tissue were WA 13.65 ± 1.65; WR 8.91 ± 1.09; CA 20.92 ± 1.46 and CR 11.02 ± 0.82 arbitrary densitometric units. There was no significant effect of temperature or diet (Table 2.3) on ACC protein abundance in mesenteric adipose tissue.

Figure 2.3 shows the levels of FAS expression, as determined by western-blot, in subcutaneous and mesenteric adipose tissues. Fatty acid synthase protein abundance in subcutaneous adipose tissue was decreased ($p < 0.05$) by feed restriction but was not affected by temperature. Similarly, in mesenteric adipose tissue, feed restriction caused a reduction ($p < 0.05$) in protein abundance of the enzyme and there was no effect of temperature on FAS protein abundance (Table 2.4).

From the enzyme activity and western-blot data, the ratios of activity to enzyme protein were calculated and the ratios were compared for animals from different treatment groups. In subcutaneous adipose tissue, ACC activity per unit of enzyme protein was lowered ($p < 0.05$) by cold environmental temperature but there was no significant effect of feeding level. In mesenteric fat, ACC activity per unit enzyme protein was not significantly altered by environmental temperature. However, there was a near-significant effect ($p = 0.06$) of feeding level on enzyme activity per unit change in enzyme protein, with dietary restriction lowering the ratio by up to 30 % compared to the *ad libitum* fed groups. There was no effect of environmental temperature or feeding level on FAS activity-protein ratios in either subcutaneous or mesenteric fat. The different enzyme activities have been plotted and linearly related against their respective protein abundance data and the relationship between the activity and protein abundance data are presented in Figures 2.4 and 2.5 for ACC and FAS respectively. The following regression

equations were obtained respectively for ACC and FAS:

$Y_{acc} = 0.053X_{acc} + 1.954$; $R^2 = 0.08$; for SC ($p = 0.19$)

$Y_{acc} = 0.024X_{acc} + 2.573$; $R^2 = 0.013$; for MS ($p = 0.59$), and,

$Y_{fas} = 0.044X_{fas} + 1.116$; $R^2 = 0.28$; for SC ($p = 0.01$)

$Y_{fas} = 0.151X_{fas} + 2.170$; $R^2 = 0.12$; for MS ($p = 0.10$).

Where $Y_{acc\ or\ fas}$ = ACC (nmol $H^{14}CO_3$ min^{-1} mg^{-1} protein) or FAS activity and $X_{acc\ or\ fas}$ = ACC or FAS protein abundance.

2.4. DISCUSSION

Animals often experience significant alterations in metabolism when exposed to a cold environment. These may include a major change in rates of fatty acid synthesis and lipid deposition in adipose tissue. There are no reported data on the activity and protein abundance of the enzymes that regulate fatty acid synthesis during cold exposure in ruminants. As part of a larger experiment reported elsewhere (Ekpe 1998), the present study was undertaken to determine whether there were adaptive changes in key enzymes of fatty acid synthesis in adipose tissues from sheep exposed to two different temperatures and fed two different levels of feed together with possible interactions of temperature and feeding level.

In the present study, the enzyme activity data show that feeding levels regulate both ACC and FAS in subcutaneous and mesenteric adipose tissues of sheep. Moreover, FAS activity in the external fat depot was significantly lower when compared to that of the internal depot. These regional variations in enzyme activities of the two fat depots are

in agreement with previous work by Anderson et al. (1972) in porcine adipose tissues and may suggest differences in metabolic activities of the different fat depots.

Furthermore, results from the individual treatment groups (WA, WR, CA and CR) showed that in the cold and warm environments ACC and FAS activities and protein abundances changed in parallel in response to feeding level. Activities and abundances were generally higher for the *ad libitum* fed groups, except in mesenteric adipose tissue of the cold-*ad libitum*-fed group where ACC protein abundance was slightly lower compared to the cold-restricted group, whereas ACC activity was higher. The feeding effect means (Tables 2.1 to 2.4), and individual treatment means (not presented) suggest that changes in enzyme protein abundance were accompanied by corresponding changes in enzyme activity (though not to the same extent in all treatments), suggesting that ACC activity and enzyme protein expression are regulated by similar mechanisms in adipose tissues. In contrast, when all the treatments were pooled together in a regression analysis there appeared to be no significant correlation between enzyme activity and the amount of enzyme protein.

In nonruminants, changes in enzyme activity are brought about, in part, by an alteration in the rate of enzyme protein synthesis, which may be a consequence of altered cellular concentration of specific mRNAs (Stapleton et al. 1990; Swierczynski et al. 1991; Girard et al; 1994; Blennemann et al. 1995). Because of the low correlation between enzyme activity and enzyme protein abundance, it is difficult to infer that the changes in enzyme activity in adipose tissues from the sheep in this study were brought about by a corresponding change in the amount of enzyme protein.

Lipogenic enzymes are known to respond to both dietary and hormonal manipulation (Hillgartner et al. 1995). The regulation of the enzymes by hormones and diet may be influenced by exposure of animals to a cold environment. The results of this study show that exposure of sheep to a cold environment leads to a decrease in activity of both ACC and FAS. Such low ACC and FAS activities are known to result in down regulation of *de novo* fatty acid synthesis in tissues. Rapid decreases in fatty acid synthesis have been reported in rats after acute (2 h) cold exposure (Buckley and Rath 1987) and in response to noradrenaline (Gibbins et al. 1985).

The mechanisms by which environmental temperature affects or regulates adipose tissue lipogenic enzymes are uncertain at the present time. However, there is evidence that during cold exposure, white adipose tissue may become more sensitized to the effect of circulating catecholamines in terms of lipid mobilization (Graham and Christopherson 1981). Plasma concentrations of free fatty acids are also known to increase after the onset of cold stress in sheep (Halliday et al. 1969) and cattle and the levels remained elevated during prolonged exposure to cold (Young 1975). Such increases in circulating catecholamines may lead to enhanced intracellular levels of cAMP and fatty acyl-CoA, both of which have been shown to inhibit ACC activity (Volpe and Vagelos 1976). Hence, a decrease in ACC activity during cold exposure of lambs may involve the participation of catecholamines in the regulation of this enzyme. In the present study, the reduced activity of ACC in adipose tissue from cold exposed sheep must have been in part due to a reduction in the catalytic efficiency of the enzyme rather than any reduction in the concentration of the enzyme protein, since no corresponding change was observed for ACC activity and its protein expression in the adipose tissues.

It is possible that decreased activity of ACC in the two adipose tissue depots from sheep exposed to a cold environment and during feed restriction was in response to the utilization of substrate to meet thermogenic requirements. There is evidence for a role for ACC in substrate utilization by tissues (Saddik et al. 1993; Lopaschuk et al. 1994). This involves the regulation of carnitine-palmitoyl-transferase-I (CPT-I, through the production of malonyl-CoA) which is an enzyme responsible for fatty acid oxidation. This decline in ACC activity may be important to remove inhibition of CPT-I and ensure the preferential use of lipogenic precursors for heat generation during exposure of the animals to a cold environment.

By design, daily feed intakes of the restricted-fed animals (WR and CR) were lower than those of their *ad libitum* counterparts (WA and CA) because the animals in the former groups were maintained at a restricted level of intakes (Ekpe 1998). In ruminants fed less than an *ad libitum* ration, there may be diurnal variations in the rate of ruminal production and in the circulating levels of short chain fatty acids (SCFA). As a result, blood levels of acetate required for fatty acid synthesis in ruminants could be low, while plasma free fatty acid levels may increase. From the point of view of the supply of carbon and reducing equivalents, dietary restriction may reduce the availability of short-chain fatty acids which are crucial for fatty acid synthesis in ruminant adipose tissue. Acetyl-CoA carboxylase would likely be directly affected by any factor which alters ruminal production of SCFA. In *ad libitum*-fed groups, however, a less direct link between the availability of SCFA and fatty acid synthesis may occur via a relationship between the rate of appearance of these acids in blood and the post-prandial increase in insulin secretion (Basset 1975; Brockman 1978). As shown by Ekpe (1998), plasma insulin

concentrations were increased in *ad libitum* compared to the restricted-fed groups. It is likely that some of the effects of feeding level on enzyme activities were mediated through its action on insulin secretion. Insulin has been shown to enhance lipogenesis from both glucose and acetate and also to inhibit catecholamine-induced lipolysis (Yang and Baldwin 1973) in ruminant adipose tissues *in vitro*. Since insulin is involved in the regulation of post-prandial changes in lipogenesis in ruminant adipose and mammary tissues (Ingle et al. 1973; Mellenberger et al. 1973), which is also regulated by the allosteric enzyme ACC, it is possible that the effects of feeding level on ACC activity were manifested, in part, by alterations in plasma insulin concentrations.

Although a high feeding level may stimulate an increase in enzyme activity through an action on insulin, the 14 % higher feed consumption by the *ad libitum*-fed animals in the cold environment (Ekpe 1998) was not enough to increase the enzyme activity above that measured for their *ad libitum* fed counterparts in the warm environment. It is possible that a large proportion of the extra feed consumed by the *ad libitum* fed group in the cold environment was used to release energy for thermogenesis rather than to provide substrate for fatty acid synthesis.

Acetyl-CoA carboxylase has been shown to be widely distributed in a number of different mammalian tissues (Thampy 1989; Iverson et al. 1990; Trumble et al. 1991). Two isoforms of ACC with molecular weights of 265,000 Da (ACC-265) and 272-280,000 Da (ACC-280) have been identified in both liver and adipose tissue. However, there has been no reported difference in ACC activity of these tissues. The relative expression of the two isoforms in ruminant tissues has yet to be determined, but data from nonruminants indicate that both isoforms may be differentially expressed in adipose

tissue. In this experiment, only one isoform of ACC, the 265 kDa, was detected in adipose tissue using peroxidase-conjugated-streptavidin. Presently, the characteristics and regulation of ACC-265 are better understood than ACC-280. ACC-265 is known to be regulated by both long and short-term control mechanisms (Kim et al. 1989).

The enzyme preparation from the two adipose tissue depots in this study exhibited low ACC activity in the absence of citrate. However, increased activity was demonstrated when assays were conducted in the presence of citrate (Figure 2.1). Based on this alteration of activity by citrate, it is possible that alteration in the phosphorylation states of ACC could be involved in its regulation in adipose tissues in sheep when they are exposed to a cold temperature and during feed restrictions. Such citrate dependence is characteristic of ACC in many animal tissues (Lane et al. 1979). In the cold exposed animals, it is impossible to explain the effect of cold exposure on the decrease in activity of ACC simply by alteration in the phosphorylation states of the enzyme but rather a combination of several other factors must also be involved since noradrenergic stimulation of white adipose tissue may also inhibit ACC activities by increasing the cytoplasmic concentration of fatty acyl-CoA.

Whereas ACC may be regulated by allosteric and (or) covalent modifications, FAS activity is not known to be regulated by such effectors. In the current study, FAS activity was generally lower in subcutaneous adipose tissue compared to the internal mesenteric adipose tissue. In both tissues, feed restriction caused a significant reduction in FAS activity in the warm environment but no difference was detected between *ad libitum* and restricted animals in the cold environment. There was no direct relationship between changes in FAS activity as a result of cold exposure and the amount of FAS

immunoprotein expression. However, when data were compared on the four treatment groups (WA, WR, CA and CR), it was observed that a change in FAS activity in any temperature x feeding level treatment combinations resulted in a parallel change in FAS protein abundance. Parallel changes in FAS activity and FAS immunoprotein were also obtained when the main treatment effect of feeding level was considered. This suggests that the feeding levels alone or in combination with environment temperature are probably more important than any single effect of environmental temperature on the regulation of the enzyme. These latter results are in agreement with current state of knowledge in the literature regarding nutritional control of FAS.

The change in FAS activity as reflected by the alteration in the amount of enzyme protein in the current study may be governed by its rate of synthesis and the stability of its mRNA (Iritani et al. 1992; Clarke 1993; Hillgartner et al. 1995). The rate of synthesis of the enzyme protein is highly dependent on the nutritional status (Moustaid and Sul 1991; Clarke and Jump 1992), hormonal environment, and the developmental states of the animal (Volpe & Vagelos 1976; Wakil et al. 1983; Hillgartner et al. 1995). Both lipogenic and lipolytic hormones participate in regulating FAS expression, and during prolonged cold exposure plasma levels of catecholamines (epinephrine and norepinephrine) and glucagon are elevated. These hormonal milieus are known to inhibit lipogenesis (Katsurada et al. 1990) and are likely to be involved in the reduction of FAS activity during cold exposure and feed restriction.

Since both insulin and plasma T_3 concentration were altered in this study (Ekpe 1998), their effects on the regulation of FAS may also be important. Changes in insulin and T_3 status that may be brought about by dietary restriction and exposure to a cold

environment may also contribute to alterations in FAS activity by affecting the FAS gene expression. Soncini et al. (1995) investigated the molecular basis for the tissue specific and nutritional/hormonal regulation of FAS gene expression. They showed increased FAS mRNA levels in adipose tissue and liver, and these were highly correlated with increased FAS activity in those tissues. Soncini et al. (1995) also observed that FAS induction in adipose tissue is at pretranslational level by increasing FAS mRNA levels in white adipose tissue.

In line with the decrease in FAS activity reported in this study, Cousin et al. 1993 showed that FAS mRNA concentration in periovarian and inguinal white fat pads of rats decreased in response to cold exposure. Blennemann et al. (1995) reported that low T_3 levels (as may be obtained during feed restriction of animals) reduced FAS mRNA compared to the euthyroid state. Thyroid hormones are thought to function by binding to nuclear receptor proteins and altering the interactions of these receptors with response element DNA sequences in the target genes, thereby changing the rate of transcription of those genes. We may conclude from these and other studies that the observed effects of cold- and diet-induced downregulation of FAS activity are as a consequence of a decrease in the tissue content of the enzyme proteins (Clarke and Jump 1992) as presented in Tables 2.2 and 2.4. Recently, Hsu et al. (1996) provided evidence for the existence of two promoters regulating FAS and suggested that promoter II activity is probably needed for low-level constitutive expression while promoter I-mediated transcription is blocked. Under lipogenic conditions, promoter II activity is minimized to remove transcription block in favor of efficient transcription of promoter I. It is unknown at present whether the ruminant FAS gene is regulated by two promoters or whether

multiple promoters will translate into multiple isozymes of the FAS enzyme.

The role of cold environmental temperature in regulating ACC/FAS activity or enzyme protein expression has not been previously studied in ruminants. The regulation of these enzymes by cold exposure and feed restriction may involve long-term control mechanisms. The primary factor in the regulation of these enzymes when animals are exposed to cold and warm temperature may be related to the overall energy balance of the animal. This regulation may be critical to the partitioning of energy to meet the thermogenic demand of those animals during such situations. The results also suggest that fatty acid synthetic rate in the adipose tissue during periods of cold exposure may be suppressed since the activities of the key enzymes that regulate this pathway were decreased following exposure to a cold environment and feed restriction.

Table 2.1. The effect of environmental temperature and feeding levels on the activity of acetyl-CoA carboxylase in subcutaneous and mesenteric adipose tissues from sheep.

	Temperature (T)		Feeding (F)		SE	T * F
Fat depot	Warm	Cold	*Ad libitum*	Restricted	(pooled)	(Prob.)
Subcutaneous fat	3.14 [a]	2.21 [b]	3.17 [c]	2.18 [d]	0.24	0.32
Mesenteric fat	3.20 [a]	2.43 [b]	3.18 [c]	2.46 [d]	0.20	0.94

ACC activity is expressed as nmol $H^{14}CO_3.min^{-1}.mg\ protein^{-1}$. Values are means of triplicate assays. Results are presented to show main treatment effects since the interactions of the main effects (temperature x feeding) were not significant. Number of animals per presented mean = 12.

SE indicates standard error of least square means.

T*F indicates probability for the interaction of temperature and feeding.

[a, b] or [c, d] Means with different superscripts within a row and within a treatment effect (temperature or feeding) are significantly different ($p < 0.05$).

Table 2.2. The effect of environmental temperature and feeding levels on the activity of fatty acid synthase in subcutaneous and mesenteric adipose tissues from sheep.

	Temperature (T)		Feeding (F)		SE	T * F
Fat depot	Warm	Cold	*Ad libitum*	Restricted	(pooled)	(Prob.)
Subcutaneous fat	2.35 [a]	1.47 [b]	2.36 [c]	1.46 [d]	0.18	0.08
Mesenteric fat	4.82	4.45	5.75 [c]	3.52 [d]	0.60	0.08

FAS assay was determined in duplicate and activity expressed as nmol NADPH oxidized per minute per mg cytosolic protein. Data are presented as least square means of the main treatment effects. Number of animal per presented mean = 12.

SE indicates standard error of least square means.

T*F indicates probability for the interaction of temperature and feeding.

[a, b] or [c, d] Means in a row and within same treatment effect (temperature or feeding) followed by different superscripts are significantly different ($p < 0.05$).

Table 2.3. The effect of environmental temperature and feeding levels on acetyl-CoA carboxylase protein abundance (densitometric units) in subcutaneous and mesenteric adipose tissues from sheep.

	Temperature (T)		Feeding (F)		SE	T * F
Fat depot	Warm	Cold	*Ad libitum*	Restricted	(pooled)	(Prob.)
Subcutaneous fat	11.28 [b]	15.97 [a]	17.29 [c]	9.96 [d]	0.92	0.06
Mesenteric fat	10.46	10.24	9.64	11.07	1.17	0.14

SE indicates standard error of least square means.

T*F indicates probability for the interaction of temperature and feeding.

[a, b] or [c, d] Means in a row and within same treatment effect (temperature or feeding) followed by different superscripts are significantly different ($p < 0.05$). Number of animals per mean = 12.

Table 2.4. The effect of environmental temperature and feeding levels on fatty acid synthase protein abundance (densitometric units) in subcutaneous and mesenteric adipose tissues from sheep.

	Temperature (T)		Feeding (F)		SE	T * F
Fat depot	Warm	Cold	*Ad libitum*	Restricted	(pooled)	(Prob.)
Subcutaneous fat	18.37	17.56	26.52 [a]	9.41 [b]	1.82	0.92
Mesenteric fat	16.60	16.08	20.16 [a]	12.52 [b]	1.14	0.29

SE indicates standard error of least square means.

T*F indicates probability for the interaction of temperature and feeding.

[a, b] Means in a common row within feeding effect and followed by different superscripts are significantly different ($p < 0.05$). Number of animals per mean = 12.

Table 2.5. Acetyl-CoA carboxylase and fatty acid synthase activities per unit of protein abundance.

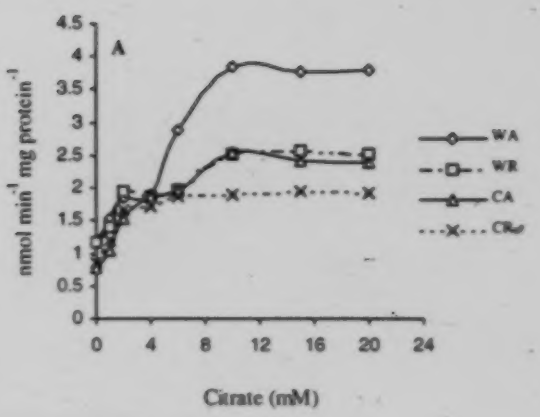
	Temperature (T)		Feeding (F)		SE	T * F
Fat depot	Warm	Cold	*Ad libitum*	Restricted	(pooled)	(Prob.)
Acetyl-coenzyme A carboxylase						
Subcutaneous fat	0.289 [a]	0.151 [b]	0.204	0.236	0.018	0.457
Mesenteric fat	0.341	0.296	0.382	0.255	0.044	0.196
Fatty acid synthase						
Subcutaneous fat	0.174	0.254	0.093	0.335	0.112	0.439
Mesenteric fat	0.287	0.398	0.294	0.392	0.133	0.211

Activities and protein abundance were determined as described under Materials and Methods. Number of animals per mean = 12.

SE indicates standard error of least square means.

T*F indicates probability for the interaction of temperature and feeding.

[a, b] Means in row within temperature effect and followed by different superscripts are significantly different ($p < 0.05$).



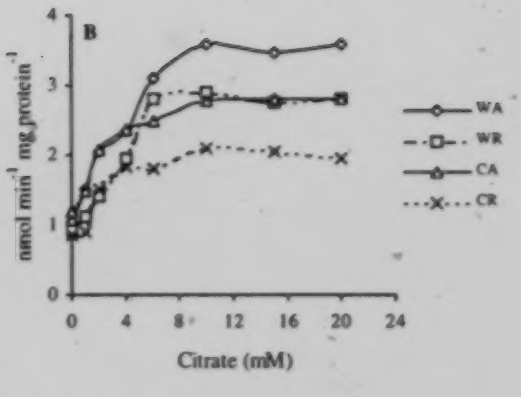


Figure 2.1. Citrate dependence of acetyl-CoA carboxylase in subcutaneous (panel A) and mesenteric (panel B) adipose tissues from sheep.

Enzyme extracts were assayed as described under Materials and Methods in the absence (0 mM) or presence of varying concentrations of citrate. WA = warm-*ad lib*, WR = warm restricted, CA = cold-*ad lib* and CR = cold restricted.

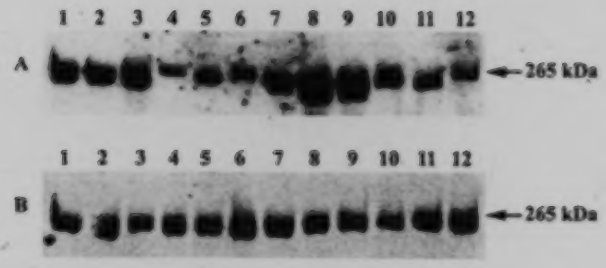


Figure 2.2. Western blot analyses of ACC protein expression in subcutaneous (panel A) and mesenteric (panel B) adipose tissues from wethers.

Western blot analyses were performed as described under "Materials and Methods". SDS-PAGE (5 % gel) was carried out by loading a 20 µg (subcutaneous) or 25 µg (mesenteric) protein sample into each lane. After transfer to nitrocellulose membrane, ACC was detected using peroxidase-labelled-streptavidin. Lanes 1-3 represent three of the animals in the warm environment and *ad libitum* feeding, lanes 4-6 represent animals in the warm environment and restricted feed intake. Lanes 7-9 are from animals on *ad lib* feed intake but exposed to cold environmental temperature while lanes 10-12 represent animals on a restricted feed intake with cold exposure. Only 12 animals are shown for illustration but all 24 animals on the various treatments were used for western-blot analysis.

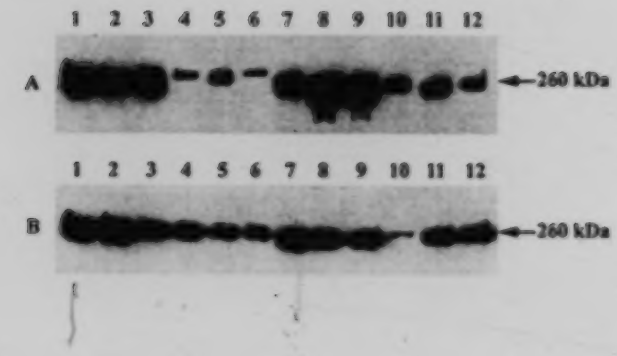


Figure 2.3. Western blot analyses of FAS protein expression in subcutaneous (panel A) and mesenteric (panel B) adipose tissues from wethers.

SDS-PAGE (5 % gel) was carried out by loading a 20 µg (subcutaneous) or 25 µg (mesenteric) protein sample into each lane. After transfer to a nitrocellulose membrane, FAS protein was detected using mouse anti-FAS monoclonal antibody and a secondary goat anti mouse-horse radish peroxidase. Lanes 1-3, 4-6, 7-9 and 10-12 respectively represent animals on warm-*ad libitum*, warm-restricted, cold-*ad libitum* and cold restricted treatments. Only 12 animals are shown for illustration but all 24 animals on treatments were used for analysis.

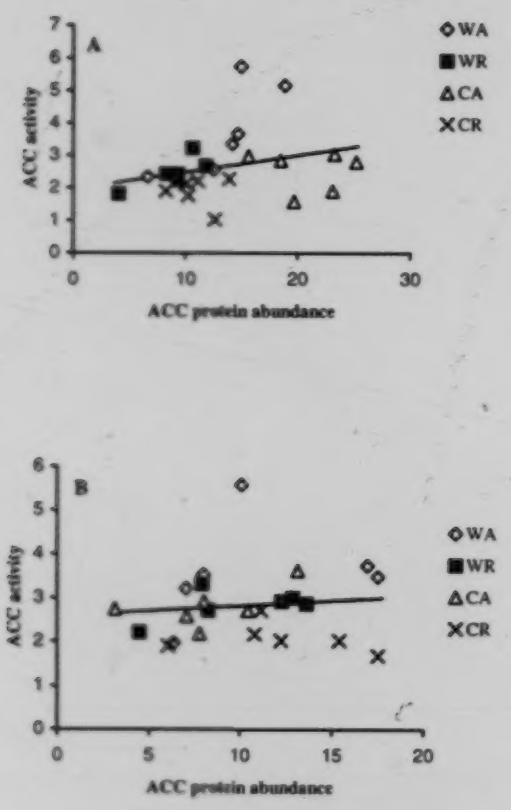


Figure 2.4. The relationship between acetyl-CoA carboxylase activity and protein abundance in subcutaneous (panel A) and mesenteric (panel B) adipose tissues from sheep. Activity and protein abundance were determined as described under "Materials and Methods". ACC activity was expressed as nmol $H^{14}CO_3$ min^{-1} mg $protein^{-1}$. Protein abundance is given as arbitrary densitometry units. Each point on the figure represents an individual animal. The regression lines were generated from least squares.

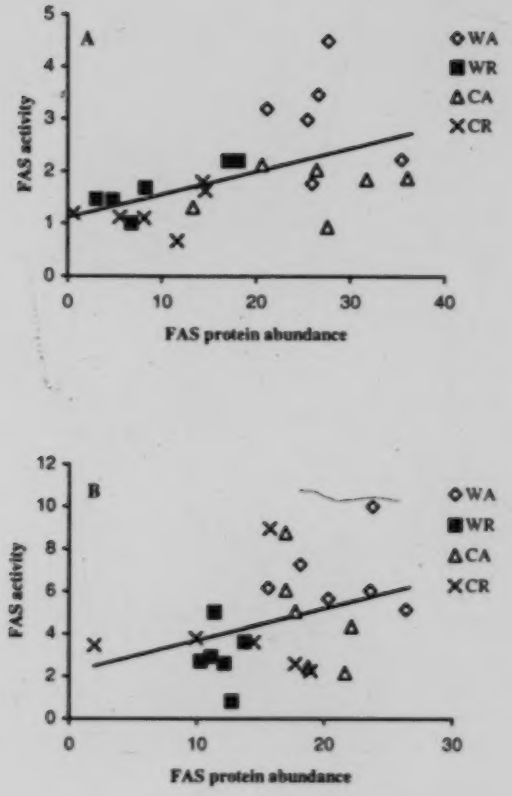


Figure 2.5. The relationship between FAS activity and protein abundance in subcutaneous (panel A) and mesenteric (panel B) adipose tissues from sheep.

Activity and protein abundance were determined as described under "Materials and Methods". FAS activity was expressed as nmol NADPH oxidized min^{-1} mg $protein^{-1}$. Protein abundance is given as arbitrary densitometry units. Each point on the figure represents an individual animal. The regression lines were generated from least squares.

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3. *In vivo* And *In vitro* Lipogenesis and Aspects of Metabolism in Ovines: Effect of Environmental Temperature and Dietary Lipid Supplementation

3.1. INTRODUCTION

The lipid content of the carcass is important for excellent organoleptic properties of meat. Excessive deposition of subcutaneous fat is, however, disadvantageous because of increased feeding costs and negative visual appeal of the carcass (Mottram and Edwards 1983). Therefore, an improved understanding of the mechanisms regulating fat synthesis may suggest ways and means by which breeding and management approaches can be utilized for improved control of lipid deposition in adipose tissue and muscle in meat animals. Adipose tissue development is related to lipogenic enzyme activity and this, in turn, is reflected in the accumulation of triacylglycerols in the adipocytes (Mendizabal et al. 1997). The fatty acids required for triacylglycerols synthesis can either be obtained from dietary sources or synthesized in the animal. Most feedstuffs used in ruminant diets are low in fat and as a consequence over 90 % of fat deposited as triacylglycerols in ruminants is synthesized by *de novo* fatty acid synthesis within the adipocytes of adipose tissue (Vernon 1986).

Regulation of substrate flow through the fatty acid synthetic pathway is imposed at two levels. Short-term changes in flow are initiated by altering the catalytic efficiency of the pace setting enzymes (Hillgartner et al. 1995). Long-term adjustments of enzyme activity occur when stimuli responsible for altering flux are sustained for prolonged periods and generally involve changes in the concentration of the regulatory enzymes. Fatty acid synthesis in adipose tissue requires the sequential action of two enzymes:

acetyl-CoA carboxylase (ACC) and the fatty acid synthase (FAS) complex. The regulation of these two enzymes in adipose tissue by nutritional and hormonal factors has been found to be basically similar to their regulation in liver. Factors that have been studied with respect to regulation of ACC and FAS include triiodothyronine, glucagon, insulin and growth factors, starvation, and feed restriction. The mechanisms by which these factors produce changes in enzyme activities have been related to alteration in the rates of synthesis of ACC and FAS enzymes and (or) changes in catalytic activity of the existing enzyme.

Dietary fat high in polyunsaturated fatty acids is another factor that is known to inhibit the activity of lipid synthesizing enzymes (Clarke et al. 1990; Clarke and Abraham 1992,), and also inhibit hepatic *de novo* fatty acid synthesis (Clarke and Jump 1992, 1993). In rats, the mechanism for this suppression is due to an inhibition of gene transcription accompanied by a decline in the corresponding mRNAs and a subsequent reduction in protein and (or) enzymatic activity of the lipogenic enzymes (Clarke et al. 1990; Clarke and Jump 1993). In ruminants, the various factors that regulate fatty acid synthesis have been studied to some length, but the underlying mechanisms have not been properly elucidated. Interactions between effects of environment and nutrition on fatty acid synthesis require further documentation in ruminants.

In the current study, we have tested the hypothesis that when ruminants are exposed to a cold environment, the rates of fatty acid biosynthesis in body tissues are reduced. A second hypothesis was that including a lipid (protected from rumen biohydrogenation) source in the animals' diet would lead to a reduction in the rates of fatty acid synthesis because of its inhibitory effect on the enzymes regulating the fatty

acid biosynthetic pathway. A third hypothesis was that both thermal exposure and dietary lipid supplementation would alter the metabolic and hormonal properties of the animal, which may result in modulation of fatty acid synthetic rates.

The study reported herein was therefore conducted to investigate the influence of different environmental temperatures together with manipulation of diet composition and feeding levels on *in vivo* and *in vitro* rates of fatty acid biosynthesis, plasma hormone concentrations and other metabolic parameters in sheep.

3.2. MATERIALS AND METHODS

3.2.1. Animals and Management

Twenty-four Suffolk-cross 5-mo old wether lambs were randomly divided into six treatment groups over two periods of experimentation. The animals were shorn and treated with Ivomec antihelmintic (0.02 ml kg^{-1}) (MSD Agzet, PointeClaire, Dugorvale, Quebec), before being transferred into individual metabolism crates where they remained throughout each period of study. Three environmental treatments (+23 °C-warm, 0 °C-cold, and, cold-pair-fed) and two dietary regimens (control and lipid-supplemented) were applied to the six experimental groups in a 3 X 2 factorial arrangement of a complete randomized design. The pair-fed groups of animals were placed on the dietary regimen at 0 °C but fed the level of feed consumed (per unit body weight) by the animals in the +23 °C (warm) environment. All animals were maintained in continuously illuminated rooms and were given *ad libitum* access to feed (except the pair-fed groups). The control diet was a barley-based diet containing 15 % crude protein (CP) and 17.24 MJ GE kg^{-1}. The lipid diet (18.22 MJ GE kg^{-1}; 14.4 % crude protein) was the control diet supplemented

with a protected lipid source (Megalac®). The gross and fatty acid composition of the two diets is shown in Tables 3.1 and A3.1, respectively. The level of nutrients in the feed was calculated to meet the requirements of growing lambs in both thermal environments (Christopherson and Young 1981; McBride and Christopherson 1984 a,b; NRC 1985). Feed was offered to the animals once daily at 0800 h, and unconsumed feed was collected and weighed daily. Water and salt blocks were continuously available to the animals. Growth of the lambs was monitored by weekly weighings. All protocols were approved by the Faculty Animal Policy and Welfare Committee, and animals were cared for according to guidelines of the Canadian Council on Animal Care (1993). A single blood sample was collected from each animal weekly using heparinized venipuncture Vacutainers (Becton Dickinson, Missassauga, ON) for determination of plasma insulin and plasma triiodothyronine. In all cases blood samples were collected between 0900 and 0930 h and before the animals were weighed.

3.2.2. Metabolic Rate and Balance Studies

Oxygen consumption measurement and 5 d balance studies were carried out during the fourth week of the study. Lambs were previously accustomed to metabolic hoods during the second and third weeks of the experiment. Oxygen consumption (V_{o2}) of the lambs was determined in the animals' acclimation environments and measured in pairs of lambs on separate days for 6 h (0930 to 1530 h) by open-circuit calorimetry (Young et al. 1975) using a dual channel paramagnetic oxygen analyzer (Taylor Servomex, Sussex, U.K.) connected to ventilated hoods (50 x 46 x 75 cm). The air flow rates were measured by a flow meter (130 L min^{-1}; Rotometer, Fisher and Porter, Warmister, PA) and were

corrected to standard temperature and pressure. The oxygen concentration difference between incoming and outgoing respired air was determined with this equipment and resulting data were acquired every 10 s and averaged over 3 min intervals by a computerized data acquisition program developed in our laboratory (Godby and Gregory 1992). The 3 min recordings were averaged over 6 h and heat production was calculated from oxygen consumption using the equation of McLean (1972).

Digestibility was determined by measuring the difference between intake and total fecal excretion (retained on a screen) of energy and nitrogen. Urinary excretion of energy and nitrogen was determined by recording daily urine output (collected in a bucket containing 10 ml 5 N hydrochloric acid). Aliquots of feed (1 %), feces (10 %) and urine (5 %) were taken from each day's collection, mixed thoroughly, and stored at –30 °C until analysis.

3.2.3. *In Vivo* Fatty Acid Synthesis

After 5 wk on the environment-feeding treatments, jugular vein catheters were inserted into individual animals for radioisotope injection. Sodium [1-^{14}C]acetate (200 μCi) in 10 ml of sterile saline was injected intravenously into each lamb, 1 h before slaughter. The injection of the radioisotope was quickly followed by a single injection of 7 ml sterile saline to flush the syringe and the needle. The isotope injection schedule was staggered 30 min apart for each animal so as to give enough time for sample processing and handling at slaughter. One hour after injection of the radioisotope, animals were killed using captive bolt stunning and were rapidly exsanguinated. *Longissimus dorsi* muscle, liver and adipose tissue (subcutaneous, mesenteric, perirenal) samples were rapidly

harvested and snap frozen in liquid nitrogen. Frozen tissues were stored at –80 °C pending analyses.

3.2.4. *In Vitro* Fatty Acid Synthesis

Adipose tissues were excised from animals within 10 min after exsanguination. Subcutaneous adipose tissue samples were taken from above the 12th and 13th ribs. Mesenteric adipose tissue was taken from duodenal site, and perirenal adipose tissue was sampled from near the left kidney. Tissue slices (~130-175 mg) were prepared free hand with a scissors and incubated in a flask in the presence of 1 μCi [1-^{14}C]acetate (Dupont NEN, Boston, MA), 10mM sodium acetate and 5 mM glucose, in 3 ml Krebs-Ringer-bicarbonate buffer (pH 7.4) containing (in mM) 119 NaCl, 4.82 KCl, 1.25 $MgSO_4$, 1.24 NaH_2PO_4, 25 $NaHCO_3$, and 2.0 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). Incubations were carried out under 95% O_2 and 5% CO_2 in a metabolic shaker for 2 h. The incubation media also contained 40 mg BSA and 0.1 unit insulin / ml buffer. Aliquots of the incubation media were counted to determine the specific radioactivity of the [1-^{14}C]acetate. Because tissue samples for *in vitro* incubations were from animals initially injected with [1-^{14}C]acetate during *in vivo* measurement, blank preparations were made that excluded the [1-^{14}C]acetate from the incubation media and this allowed correction for tissue background radioactivity. All *in vitro* incubations lasted 2 h and reactions were terminated by addition of 0.1 ml of 70% perchloric acid. Following the termination of incubation, tissue samples were removed from incubation media, rinsed in non-radioactive buffer, placed in microfuge tubes and snap frozen in liquid nitrogen. All samples were kept at – 80 °C pending analyses.

3.2.5. Lipid Extraction

Frozen tissues were ground into powder in liquid nitrogen, and 0.2 g powdered adipose tissue or 1 g pulverized liver or muscle samples were weighed into 50 ml tubes with teflon lined screw caps. Samples were homogenized in 20 ml chloroform: methanol (2:1 v/v; Folch et al. 1957) with a polytron homogenizer for extraction of total lipids. Homogenate samples were kept overnight at 37 °C in a metabolic shaker and then transferred into stoppered graduated cylinders, and equilibrated with 5 ml of 0.88 % NaCl solution. After separation of the mixture into two phases, the upper methanol phase was aspirated and aliquots of the bottom chloroform layer were collected into pre-weighed 20-ml glass scintillation vials and evaporated to dryness under N_2. The weight of the lipid extract was determined by difference and expressed as percentage of weight of sample homogenized. Dried samples were resuspended in 10 ml liquid scintillation cocktail (containing 16 g of 2, 5- Diphenyloxazole and 0.4 g of 1, 4-bis[2-(5-phenyloxazolyl)] benzene in 4 liters of Toluene), and radioactivity was counted with Packard 1600CA TRI-CARB® scintillation analyzer (Packard Instrument Co., Downers Grove, IL. U.S.A.). Incorporation of ^{14}C-labeled acetate into total lipids was calculated as disintegrations per minute (dpm) per gram wet tissue for the *in vivo* experiment, and as nmol [^{14}C]acetate incorporated per 2 h per 100 mg wet tissue for the *in vitro* experiment.

3.2.6. Hormone Analyses

Plasma insulin and plasma total triiodothyronine (T_3) were determined following validation for sheep plasma and checking parallelism using radioimmunoassay kits (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA).

3.2.7. Nitrogen and Other Analyses

Samples of feces and urine were freeze-dried and their energy content determined by bomb calorimetry (Parr instrument Co., IL, U.S.A.). Nitrogen content was determined by the macro-Kjeldahl method (Association of Official Agricultural Chemists 1990).

3.2.8. Statistical Analyses

All data were analyzed as a 3 x 2 factorial design using the GLM procedure of the Statistical Analysis System Institute (SAS 1996). The model included Period, Environment (cold, cold-pair-fed and warm), Diet, Diet x Environment and Period x Diet x Environment. The separate interactions of environment and diet with period (that is, Period x Environment and Period x Diet) were pooled together with the standard error estimate. The least square mean for period was tested against error means square while Environment, Diet and Environment x Diet were tested using mean square for Period x Environment x Diet as the error term (model I). In cases where the period effect was not significant, data were reanalyzed as a completely randomized design with model statement containing Environment, Diet, and Environment x Diet (model II). In both models and where significant differences were observed, comparisons were made using the least significant difference procedure of SAS. Level of significance was set at $p < 0.05$. All data are presented as least square means together with their pooled standard error.

3.3. RESULTS

3.3.1. Feed Intake, Weight Gain and Body Fat

There was a significant effect of environment and diet on feed intake of the animals as presented in Table 3.2. Feed intake was increased ($p < 0.05$) by exposure of the animals to a cold environment (except the pair-fed groups) when compared to animals in the warm environment. Lipid supplementation of the diet caused a reduction ($p < 0.05$) in feed intake of the animals. There was no significant interaction ($p > 0.05$) between the effects of environment and diets on intake of the animals. Feed efficiency of the animals was not affected ($p > 0.05$) by environment or dietary lipid supplementation but numerically higher values were calculated for animals in the cold compared to the warm environment. Similarly, lipid supplementation gave a higher numeric but non-significant value ($p > 0.05$) for feed efficiency compared to the control diet.

Average daily gain (ADG) was significantly increased ($p < 0.05$) in the cold-exposed animals compared to those in the warm environment. Average daily gain of cold-exposed animals was about 38 % higher than in WA (warm-acclimated) animals. Average daily gain of COPF (cold-pair-fed) animals was, on average, 21 % higher compared to WA animals, but this was not a significant difference. There was no effect ($p > 0.05$) of dietary lipid supplementation on average daily gain of the animals. As shown in Figure 3.1, the relationship between average daily gain and feed intake was linear and significant ($p = 0.05$) only in the warm environment.

Ingesta-free body weight was significantly increased ($p < 0.05$) in cold-exposed animals compared to COPF and WA groups. Ingesta-free body weight was numerically

similar for both COPF and WA animals. There was no significant effect ($p > 0.05$) of dietary lipid supplementation on the ingesta-free body weight.

3.3.2. Plasma Hormone Concentrations

Plasma insulin concentrations significantly increased from week one of the experiment until the animals were slaughtered. Animals fed the control diets in the two environments had higher plasma insulin concentrations when compared to animals that were fed the lipid supplemented diet. This reduction in plasma insulin concentration as a result of feeding a fat diet approached significance ($p = 0.07$). Cold exposure tended to increase plasma insulin concentration but the effect was not significant. There were no significant interactions of environment, diet and time on plasma insulin concentrations. Weekly changes in plasma T_3 concentration followed a sigmoidal pattern throughout the experiment. There was no influence of environment or diet or their interactions on its levels. However, T_3 concentration was numerically higher in the cold environment and during control feeding.

3.3.3. Metabolic Rate

Metabolic heat production expressed in MJ d^{-1} $kg^{-0.75}$ was significantly ($p < 0.05$) affected by both environment and dietary lipid supplementation (Table 3.3). Heat production (HP) was higher ($p < 0.05$) in animals exposed to the cold environment (including the pair-fed groups) when compared to those in the warm environment. On the average, HP of animals in the cold environment was 40% higher compared to those of the animals in the warm environment. Animals on the control diet also had higher ($p < 0.05$) rates of HP

compared to those on the fat diet. The interaction between environment and diet on HP of the animals was highly significant ($p = 0.001$) resulting in a decrease in HP in the cold environment and an increase in HP in the warm environment in animals on lipid supplemented diet compared to the control. Regression analysis of HP on metabolizable energy intake (MEI) showed that the relationship was not significant. In the cold environment, the regression equation was, HP = - 0.06 + 0.33 (MEI); $R^2 = 0.08$, $p = 0.29$, and in the warm environment the equation was, HP = 0.072 + 0.13 (MEI); $R^2 = 0.10$, $p = 0.45$. These equations indicate that any increase in MEI in the warm environment was probably not diverted to heat production to the same extent as it is in the cold environment.

3.3.4. Balance Studies

3.3.4.1. Energy Balance

Urinary energy output (MJ d^{-1} kg^{-1}) by animals in the cold environment was similar to that by animals in the warm environment (Table 3.3). However, urinary energy output was significantly higher in the cold-*ad libitum* group compared to the cold-pairfed group. Dietary lipid supplementation decreased ($p < 0.05$) urinary energy output and there was no environment by diet interaction. Fecal energy output by the animals was significantly affected by environment and by diet. In analyzing the data on fecal energy output, it was noticed that period effect in the general linear model procedure was significant and consequently the mean square value for Period x Environment x Diet was used as an error estimate for testing Environment, Diet and Environment x Diet interactions. With this error term, there were no significant interactions of environment and diet on fecal

energy output of the animals. However, dietary lipid supplementation decreased ($p < 0.05$) fecal energy output while cold exposure had the opposite effect ($p < 0.05$). When fecal energy was expressed as MJ d^{-1} $kg^{-0.75}$ the effects of treatments were no longer significant.

Values for energy balance (EB), calculated as the difference between MEI (MJ d^{-1} $kg^{-0.75}$) and HP (MJ d^{-1} $kg^{-0.75}$) of animals are shown in Table 3.3. Neither the main variables nor the interaction of environment and diet had any effect on EB ($p > 0.05$). Although the cold-pairfed animals had numerically lower values compared to their counterparts in the warm environment (0.716 for COPF versus 0.883 MJ d^{-1} $kg^{-.75}$ for WA).

The relationship between EB and MEI was best described by a linear regression as shown in Figure 3.2. In the cold environment there was a significant relationship between EB and MEI as described by the following equation:

$EB_{cold} = 0.060 + 0.672$ (MEI); $R^2 = 0.27$, $p = 0.04$.

In the warm environment the relationship between energy balance and MEI was highly significant ($p = 0.001$) as given by the following equation:

$EB_{warm} = -0.072 + 0.873$ (MEI); $R^2 = 0.84$, $p = 0.001$.

3.3.4.2. Nitrogen Balance

Patterns of N intake and urinary N output were similar to the pattern of feed intake. Nitrogen intake was significantly higher ($p < 0.05$) in the cold compared to the warm environment but there was no significant difference between the cold pair-fed groups and the animals in the warm. Cold exposure increased ($p < 0.05$) but dietary lipid

supplementation decreased ($p < 0.05$) urinary N output (g d^{-1}) by the animals. There was no significant difference in the urinary N output by animals in the pair-fed groups in the cold when compared to the warm *ad libitum* fed groups. When urinary N output was expressed as g d^{-1} per unit metabolic body weight, the effect of environment was not significant whereas dietary lipid depressed ($p < 0.05$) N output. Both environmental temperature exposure and dietary lipid supplementation significantly affected fecal N (g d^{-1}) output. Fecal N output was significantly elevated in animals maintained in the cold (including the pair-fed groups) compared to the warm. Dietary lipid supplementation reduced ($p < 0.05$) fecal N output in the animals with significant interaction of environment and diet.

3.3.5. *In Vivo* and *In Vitro* Fatty Acid Synthesis

In this experiment, the rates of incorporation of [1-^{14}C]acetate was measured as an index of fatty acid synthesis *in vivo*, and the results are shown in Table 3.4. Exposure of the animals to a cold environment and supplementing their diets with a protected lipid source had no effect ($p > 0.05$) on *in vivo* [1-^{14}C]acetate incorporation in either *longissimus dorsi* muscle or liver. In subcutaneous adipose tissue, there was a diet and environment interaction ($p = 0.03$). Cold exposure reduced [1-^{14}C]acetate incorporation when the control diet was fed *ad libitum* and lipid supplementation decreased [1-^{14}C]acetate incorporation in the warm environment. Furthermore, the main effects of diet on subcutaneous fat were significant ($p = 0.02$) while that of environment only approached significance ($p = 0.07$). The apparent rate of fatty acid synthesis in mesenteric adipose tissue depot was depressed ($p < 0.05$) by lipid supplementation except when the animals

were pair-fed in the cold environment as indicated by a diet x environment interaction ($p < 0.05$). In perirenal adipose tissue, dietary lipid supplementation decreased but cold exposure increased ($p < 0.05$) the rates of [1-^{14}C]acetate incorporation.

In vitro in subcutaneous adipose tissue (Table 3.5), the rates of fatty acid synthesis in tissues from the cold-exposed animals were not significantly different than in tissues from animals that were maintained in the warm environment. Cold exposure reduced the rate of [1-^{14}C]acetate incorporation into fatty acids in mesenteric and perirenal adipose tissues when the two diets were fed *ad libitum* but the effect was not significant. Tissue from the pair-fed groups however had significantly higher ($p < 0.05$) rates of fatty acid synthesis when compared to tissue from the *ad libitum* groups in both CO and WA environments. In all three adipose tissue depots studied *in vitro*, dietary lipid supplementation reduced ($p < 0.05$) their rates of fatty acid synthesis when the animals were maintained in the warm environment. *In vitro* perirenal and mesenteric adipose tissue had lower incorporation of acetate into lipids when compared to subcutaneous adipose tissue. *In vitro* rates of incorporation of ^{14}C into lipids in perirenal and mesenteric fat were not different from each other.

3.3.6. Total Lipid Content of Skeletal Muscle, Liver and Adipose Tissues

The total lipid composition (g/100 g tissue) of *longissimus dorsi* muscle, liver and adipose tissue is presented in Table 3.6. The average total lipid content of *longissimus* muscle was 3.53 %, liver 5.76 %, and subcutaneous 71.74 %, mesenteric 84.03 % and perirenal adipose tissue, 88.51 %. Lipid content of the internal fat depots was on average 20 % higher than in the peripheral (subcutaneous) adipose tissue depots. There was no

effect of environmental temperature or diet on lipid content of sheep tissues in the current study. There was also no interaction of environment and diet on total lipid content of the tissues.

3.4. DISCUSSION

3.4.1. Feed Intake, Weight Gain and Average Daily Gain

In *ad libitum*-fed groups, feed was made available to appetite at all times and enough feed was provided to ensure that there was always about 5 % feed refusal. No problems were experienced with the animals' acceptance of the rations offered. By design, two groups of animals were pair-fed to intakes of their counterparts in the warm environment, in order to remove any confounding effects of temperature and feed intake in the cold environment. As a result, feed intakes of the animals in these pair-fed groups were not different from those of the animals in the warm environment. Cold exposure dramatically increased ($p < 0.05$) feed intake in the *ad libitum* fed groups which is in agreement with previous findings (Graham et al. 1959; Ames and Brink 1977; Kennedy 1985; Sano et al. 1995). It is possible that feed intakes in the cold environment were increased to compensate for increased passage rate in the gastrointestinal tract and reduced digestibility of feed in this environment. The enhanced feed intakes may also have been necessary to provide substrates for oxidation to meet the increased heat requirement of animals in the cold environment.

The lower average daily gain (ADG) by sheep in the warm compared to the cold environment could have resulted from differences in feed intakes by the animals in these environments since the efficiency of feed utilization was similar across treatments. Using

regression analysis ADG was linearly related to feed intake as described by the relationship depicted in Figure 3.1. Based on the regression equations based on data from animals in the cold and warm environments it seems evident that ADG was higher at nearly all intakes in the cold environment. Average daily gain values in this study are in agreement with published data on weight change of 0.33 – 0.43 kg d^{-1} in sheep (NRC 1985). Average daily gain values were up to 50 % higher compared to values of 0.14 – 0.24 kg d^{-1} reported by McBride and Christopherson (1984b) for 10 wk old lambs maintained in cold and warm environments. The lower ADG in that study was likely related to the younger age of the animals, diet type and duration of exposure to the environments.

The higher feed efficiency observed in the cold environment and in lipid supplemented diet (though not significantly different) suggested that feed intakes were better utilized. The general relationship of feed efficiency ($F_{eff.}$) on intake (FI) appeared to be curvilinear when data from all animals on the study were combined, although the regression equation ($F_{eff.} = - 0.007(FI)^2 + 0.0064(FI) - 1.304$; $R^2 = 0.16$, $p = 0.56$) was not significant. In the cold, the regression equation was $F_{eff.} = 0.272 - 0.001(FI)$, $R^2 = 0.004$, $p = 0.81$, and in the warm environment the equation was, $F_{eff.} = - 0.001(FI)^2 + 0.106(FI) - 2.23$, $R^2 = 0.59$, $p = 0.11$. Neither of these equations was significant.

For the purpose of analysis, the metabolizable energy contents of the two diets were calculated from digestible energy obtained in balance studies multiplied by a factor of 0.8. The slopes of the regression lines representing the relationship between EB and MEI (Figure 3.2) suggest that up to one-third of MEI may not be available to the animal in the cold environment whereas in the warm environment there could be better energy

retention given the equations presented in Figure 3.2. This could be due to depression in metabolizability of diets at high levels of intake, and also the fact that digestible energy may be used with different efficiencies in the two environments due to heat increment associated with higher levels of feed intake in the cold.

3.4.2. Metabolic Rates and Balance Studies

As expected, heat production (HP) by the cold acclimated wethers was higher than that of the warm-acclimated animals, in agreement with previous studies in cattle (Miaron and Christopherson 1992). The observed elevation of HP in cold-acclimated animals might, in part, be due to increased non-shivering thermogenesis (Miaron and Christopherson 1992). Reduced HP by wethers in the warm environment in this study was probably not caused by reduced energy intake since no feed refusal occurred and intake was similar to pair-fed animals in the cold environment. Since HP by the pair-fed animals in the cold was elevated compared to animals in the warm environment, it is possible that some of the metabolizable energy was diverted away from tissue growth to support thermogenesis. This was reflected by similar energy balances of the animals in the warm-*ad libitum* and cold-*ad libitum* groups even though a higher feed intake occurred in the cold group. Another factor that could contribute to elevated HP in the cold environment is the effect of gut fill and liver metabolism as a result of increased feed intake. In sheep, gut and liver metabolism decreased rapidly over 21 d following a reduction in feed intake (Burrin et al. 1989; Freetly et al. 1995). In cattle, gut and liver metabolism also accounted for 35-55 % of the whole animal oxygen usage (Reynolds et al. 1986; Eisemann and Nienaber 1990). This suggests that an increased gut fill in *ad libitum*-fed sheep in the

cold environment in this study could have resulted in increased gut and liver metabolism which would contribute to increased HP measured in animals in this environment. This cold induced thermogenesis may therefore be a homeostatic response that will enable the animals to increase HP using substrates derived from dietary metabolizable energy or through mobilization of body tissues.

The results of this study showed that N excretion decreased when N intake decreased and increased as intake increased when the animals were in warm or cold environments. These results are in agreement with those of Freetly and Nienaber (1998) who showed that in mature cows the pattern of N excretion and elimination decreased and increased when intakes of N decreased and increased, respectively. Despite increased N excretion associated with increased N intake in the cold environment in this study, N retention was also increased in the cold *ad libitum*-fed group compare to the warm environment. This increased N retention is consistent with higher ADG of the animals maintained in the cold environment.

3.4.3. Plasma Hormone concentrations

In this study, plasma T_3 concentration was not significantly affected by either cold exposure or diet fat supplementation. This is contrary to earlier findings (Kennedy et al. 1977; Miaron and Christopherson 1992) that prolonged exposure to cold increased plasma T_3 concentration in steers. The small increases observed in plasma insulin levels during cold exposure are consistent with similar increases observed in cold-adapted heifers (Scott and Christopherson 1993). However, Sasaki et al. (1982) showed unaltered

basal insulin levels for sheep in a cold environment. It is possible that the impact of temperature on insulin levels may be influenced by level of feeding and perhaps diet.

There is evidence (Smith et al. 1986) that cold acclimation results in increased sensitivity to insulin in brown adipose tissue and this may also hold true for white adipose tissue. Despite recent advances in understanding intracellular signaling, the mechanisms by which insulin regulates metabolic processes are still unclear and the mechanisms by which insulin brings about its major effects on metabolism such as fatty acid synthesis have yet to be elucidated (Moule et al. 1995). However, interaction of insulin with its receptors at the cell surface stimulates intrinsic tyrosine kinase activity of the receptors which increases tyrosine phosphorylation of a number of intracellular substrates (Sun et al. 1991) which may affect fatty acid synthesis. In nonruminants, insulin is thought to increase the rate of fatty acid synthesis from glucose by stimulating the rate of glucose transport into the cell (Gould and Holman 1993). In ruminants, glucose is not a major substrate for the fatty acid biosynthetic pathway, and so, the effect of insulin on fatty acids biosynthesis may involve only activation of key enzymes in the pathway for fatty acids biosynthesis.

3.4.4. *In Vivo* and *In Vitro* Fatty Acid Synthesis

The quantity of fat deposited in ruminant animals is a reflection of both the cellular synthesis and uptake of fatty acids of dietary origin as well as triacyglycerol mobilization. Thus, the *in vivo* data demonstrated that fatty acid synthesis occurred at a higher rate in the adipose tissue of sheep compared to the other tissues studied. The occurrence of fatty acid synthesis in adipose tissue (rather than liver) in ruminant animals is quantitatively

important because the liver can then be devoted to the production of glucose in these species.

The contributions of different adipose tissues to total lipogenesis may vary in ruminants because this tissue is nonhomogenous in terms of metabolism. Based on *in vitro* measurements, the current study provided evidence that subcutaneous adipose tissue may be relatively more active at incorporating [^{14}C]acetate into tissue lipids compared to the internal adipose tissue depots. These regional differences in the contribution of adipose tissue depots are in agreement with the results of Ingle et al. (1972) which showed that, in mature sheep and market steers, the capacity for fatty acid synthesis was two- to threefold higher in subcutaneous depots than internal adipose tissue depots. In contrast, other studies (Vernon 1980, 1986) have reported different rates of lipogenesis with the internal fat depots incorporating greater amounts of acetate into lipids compared to the peripheral adipose tissue depots. Ingle et al. (1972) suggested that differences in fatty acid synthesis rates between adipose tissue sites within same animal may be due to differences in cell numbers, and possibly due to different growth rates of the adipocytes.

The higher apparent rates of fatty acid synthesis in adipose tissues of cold-exposed compared to warm-exposed animals were probably caused by enhanced rates of lipid turnover to meet the thermogenic demands of animals in the cold environment. The fact that there were increased rates of *in vivo* fatty acid synthesis in the cold environment in all adipose tissue depots is consistent with the increased feed intake and relatively better feed efficiency of animals in this environment. The increased rates of fatty acid synthesis reported in cold-exposed sheep in this experiment are in agreement to those reported by Trayhurn (1981) in white adipose tissue from cold-acclimated mice. This

increase in rate of [1-^{14}C]acetate incorporation in the cold is likely to be a consequence of the increased requirement for substrates for thermogenesis. During long-term exposure to a cold environment when fatty acid oxidation may be enhanced the incorporation of labeled isotope into fatty acids may also be increased because of increased turnover (Nicholls and Locke 1983). Thus, the increase in lipid synthesis observed during cold exposure in the current study could partially be explained by an increase in activity of ACC and FAS both of which regulate fatty acid turnover (Buckley and Rath 1987).

The low rates of *in vivo* fatty acid synthesis observed in the liver are consistent with the view that this tissue synthesizes fatty acids at relatively low rates in ruminants. An even lower rate than in liver was observed in the skeletal muscle (*longissimus dorsi*) which is known to be traditionally nonlipogenic. Fatty acid synthesis in this tissue might be due to the lipogenic activity of associated intramuscular adipose tissue. There was no significant effect of environment or diet, or their interaction on the apparent rates of fatty acid synthesis in either the liver or the skeletal muscle.

Adipose tissue is extremely sensitive to changes in nutritional status. Thus, the regulation of its lipogenesis in response to diet may be similar to the regulation reported in the liver of other species. It is probable that rapid changes in acetyl-CoA carboxylase activity, which results in changes in concentration of malonyl-CoA, regulate the rates of fatty acid synthesis (Buckley and Rath 1987) in both the liver and adipose tissue.

As presented in Table 3.6, the total lipid content observed in *longissimus dorsi* (LD) muscle in this study was, on average, relatively less than a range of values (4.85 – 5.15 g / 100 g tissue) reported by Solomon et al. (1991) in ram lambs fed rapeseed meal or whole rapeseed or soybean meal. The difference in total fat content of muscle between

these two studies could relate to differences in age of the animal at time of slaughter. In the current study, the wether lambs were slaughtered at 45-kg liveweight, whereas the slaughter weight was 70-kg liveweight in the study reported by Solomon et al. (1991). Notwithstanding, the lipid content (g/100 g) of adipose tissue was, in general, similar to or higher than values reported by Solomon et al. (1991).

3.4.5. Effect of Dietary Fat on Fatty Acid Synthesis

In subcutaneous adipose tissue dietary fat supplementation depressed the apparent rates of fatty acid synthesis in animals in the warm but not in the cold environment. Both in mesenteric and perirenal adipose tissues *in vivo*, dietary fat supplementation depressed the rates of [^{14}C]acetate incorporation into fatty acids in both warm and cold environments. These apparent rates of fatty acid synthesis in adipose tissue were paralleled by similar measurement in the *in vitro* system only in tissues from warm exposed animals.

This study indicated that dietary lipid when fed to wethers resulted in a depression in lipid synthetic rate. This conclusion is in agreement with Hood et al. (1980) who showed that the rates of fatty acid synthesis in ovine tissues were suppressed by feeding a protected lipid source. It is not known for certain whether the polyunsaturated fatty acids in the diet in the current experiment were exerting an inhibitory effect on the rate of fatty acid synthesis *in vivo*. However, diets which are high in lipid have been shown to depress the rates of fatty acid synthesis in mammalian tissues (Clarke et al. 1990; Clarke and Jump 1992). The mechanisms by which the inhibition occurs are still not clear but there is evidence in liver from nonruminant species that such mechanisms may involve the

suppression of the expression of enzymes involved in fatty acid biosynthesis (Cheema and Clandinin 1996).

The basic requirement for a dietary fat to inhibit expression of lipogenic proteins is that it contains 18 carbons and possesses at least two conjugated double bonds in the 9, 12-position (Emken et al. 1987). The inhibitory potency of dietary polyunsaturated fatty acids on adipose tissue lipogenesis varies depending on the degree of unsaturation and chain length (Clarke et al. 1990; Clarke and Jump 1992). Obviously, since dietary fats vary in their polyunsaturated fatty acid content they will also differ in their capacity to inhibit lipogenesis. Analyses of the diets used in this study showed that the fat supplemented diet has more unsaturated fatty acids than the control diet (Appendix 3, Table A3.1) and so the inhibitory action of this diet on fatty acid synthesis in adipose tissue may be due in part to this component.

The regulatory control of adipose tissue lipogenesis in ruminants by dietary fat may be not solely due to the concentrations of polyunsaturated fatty acids in the diet since saturated fatty acids have been shown to be equipotent (Clarke and Jump 1993) in reducing lipogenesis in rats. As dietary fat suppresses adipose tissue fatty acid synthase activity and rates of fatty acid biosynthesis (Clarke and Jump 1993), the results of this study therefore suggest that the synthesis of FAS protein in adipose tissue may be the point of regulation of adipose tissue lipogenesis.

In summary, these results indicate that adipose tissues from different anatomical sites incorporate [1-^{14}C]acetate into fatty acids at different rates. The apparent rates obtained in the liver, skeletal muscle and adipose tissue indicate that the latter is the major site of fatty acid synthesis in the sheep. The study also concludes that there was no

effect of environment or diet on rates of fatty acid synthesis in skeletal muscle and liver. The three adipose tissue depots however, responded in different manners to the treatments. *In vivo*, with internal adipose tissue depots, dietary lipid reduced the apparent rates of fatty acid synthesis both in the cold and warm environments. Within peripheral adipose tissue depots, dietary lipid had no effect in the cold but significantly reduced the rates of fatty acid synthesis in the warm environment.

Table 3.1. Dietary composition of control and fat supplemented diets.

Ingredients [z]	Control diet kg / 1000 kg	Fat diet kg / 1000 kg
Alfalfa hay	198.7	200.3
Rolled barley	692.3	626.8
Megalac[y]	---	63.08
Soybean meal	99.4	100.1
Vitamin premix[x]	2.85	2.87
$CaCO_3$	4.45	4.49
Trace mineralized salt[w]	2.12	2.13
Vitamin E	0.23	0.24
Gross Energy (MJ kg^{-1})	17.24	18.22
Dry Matter %	90.91	90.68
Crude protein %	14.99	14.35

[z]As fed basis

[y]Rumen by-pass fat; registered trade mark of VOLAC Ltd. and licensed to Church and Dwight Co., Inc. Don Mills, Ontario, Canada. Minimum total fat 82.5%; Minimum fatty acids, 56.0%; Maximum Unsaturated fatty acids, 43.8%; Maximum FFA, none; Maximum triacylglycerols, 25.4%; Equivalent minimum of calcium salts of fatty acids 60.4%; Maximum moisture 7.0%; BHT as preservatives.

[x]Vitamin premix contained vitamin A 10,000,000 IU / kg; vitamin D_3 1,000,000 IU / kg; and vitamin E 10,000 IU / kg and manufactured by PMT Inc. Regina, SK. Canada. Other ingredients on the label include wheat shorts, wheat middlings, rice hulls, calcium carbonate, iron oxide, mineral oil and ethoxyquin.

[w]Low copper.

Table 3.2. The effect of environmental temperature and diet lipid on feed intake (FI), average daily gain (ADG), body fat at slaughter, plasma insulin and triiodothyronine (T_3).

	Environment (E)[z]				Diet (D)[y]			E*D[x]
	CO	COPF	WA	SE	CTL	LIPID	SE	Prob.
FI ($g\ d^{-1}\ kg^{-.75}$)	126[a]	107[b]	108[b]	2.61	120[e]	107[f]	2.13	0.33
$F_{eff.}$ (kg/kg)	0.23	0.24	0.20	0.02	0.21	0.24	0.02	0.68
Weights at start *(kg)*	27.6	26.6	26.9	0.85	27.6	26.5	0.70	0.61
ADG (kg)	0.42[a]	0.37[ab]	0.30[b]	0.02	0.37	0.36	0.02	0.56
IF_{BW} (kg)	28.75 [a]	25.95 [b]	25.96 [b]	0.89	27.13	26.64	0.73	0.71
Insulin (μIU/ml)	26.56	22.00	23.50	2.37	26.27	21.78	1.93	0.86
T_3 nmole/L	2.86	2.74	2.64	0.19	2.85	2.65	0.15	0.36

Values are least square means. Number of animals = 8 in environment and 12 in diet effects.

$F_{eff.}$ = Feed efficiency (kg gain per kg intake)

IF_{BW} = Ingesta-free body weight

[a, b, c] or [e, f] Means in common row and within same treatment effect (environment or diet) and followed by different superscripts are significantly different ($p < 0.05$).

[z]CO = Cold environment, COPF = cold pair-fed, and, WA = warm environment.

[y]CTL, LIPID are respectively control or lipid supplemented diet.

[x]E*D = Interaction of environment and diet. SE = Standard error of the least square means.

Table 3.3. Effect of environmental temperature and diet on heat production (HP), and nitrogen and energy balances in sheep.

	Environment (E)[z]				DIET (D)[y]			E*D[x]
	CO	COPF	WA	SE	CTL	LIPID	SE	(prob.)
HP ($MJd^{-1}kg^{-0.75}$)	0.31[a]	0.28[a]	0.21[b]	0.02	0.30[e]	0.24[f]	0.01	.0001
[w]UE ($MJd^{-1}kg^{-0.75}$)	0.059[a]	0.053[b]	0.056[a,b]	0.002	0.06[e]	0.05[f]	0.001	0.22
[v] FE ($MJ\ d^{-1}$)	10.45[a]	9.82[a]	8.51[b]	0.33	10.30[e]	8.89[f]	0.27	0.15
[v]FE ($MJ\ d^{-1}kg^{-0.75}$)	0.67	0.67	0.59	0.03	0.68	0.61	0.02	0.28
[u]EB ($MJ\ d^{-1}kg^{-0.75}$)	0.872	0.716	0.883	0.09	0.811	0.837	0.07	0.86
N-intake $g\ d^{-1}$	43.60[a]	35.88[b]	34.70[b]	0.90	41.27[e]	34.85[f]	0.74	0.64
Urinary-N ($g\ d^{-1}$)	17.17[a]	13.31[b]	13.82[b]	0.72	16.55[e]	12.98[f]	0.59	.08
Urinary- N ($g\ d^{-1}\ kg^{-0.75}$)	1.09	0.92	0.98	0.05	1.08[e]	0.91[f]	0.04	0.30
Fecal N $g\ d^{-1}$	14.97[a]	14.31[a]	12.33[b]	0.47	15.49[e]	12.25[f]	0.39	0.04
N-balance $g\ d^{-1}$	12.62	10.32	11.31	0.89	11.22	11.61	0.72	0.85

Values are least square means of main treatment effects. Environment n = 8, diet n = 12.

[a, b, c] or [e, f] Means in common row and within same treatment effect (environment or diet) and followed by different superscripts are significantly different ($p < 0.05$).

[z]CO = cold environment, COPF = cold pair-fed, and, WA = warm environment.

[y]CTL, LIPID are respectively control or lipid supplemented diet.

[x]E*D = Interaction of environment and diet. SE = standard error of the least square means.

[w]Urinary energy

[v]Fecal energy

[u]Energy balance

Table 3.4. Effect of environmental temperature and dietary lipid supplementation on *in vivo* fatty acid synthesis (dpm / g tissue) in adipose tissue, muscle and liver from sheep.

	Environment (E)[z]				DIET (D)[y]			E*D[x]
Tissue	CO	COPF	WA	SE	CTL	LIPID	SE	Prob.
Longissimus dorsi	665	494	620	108	537	648	90	0.23
Liver	2491	2923	2138	370	2570	2465	292	0.46
Subcutaneous fat	10482	11618	9866	1121	11795 [e]	9514 [f]	884	0.03
Mesenteric fat	10854	8219	9063	1061	10722 [e]	8035 [f]	895	0.02
Perirenal fat	10043 [a]	8627 [a]	6043 [b]	900	10269 [e]	6206 [f]	710	0.01

Values are least square means of main treatment effects. Number of animals = 8 for each mean under environment and 12 for each mean under diet effect.

[a, b] or [e, f] means in common row and within same treatment effect (environment or diet) and followed by different superscripts are significantly different ($p < 0.05$).

[z]CO = cold environment, COPF = cold pair-fed, and, WA = warm environment.

[y]CTL, LIPID are respectively control or lipid supplemented diet.

[x]E*D = Interaction of environment and diet. SE = standard error of the least square means.

Table 3.5. Effect of environmental temperature and dietary lipid supplementation on *in vitro* fatty acid synthesis (nmol [^{14}C]acetate/ 2 h/ 100mg wet tissue) in adipose from sheep.

	Environment (E)[z]				DIET (D)[y]			E*D[x]
Adipose Tissue	CO	COPF	WA	SE	CTL	LIPID	SE	Prob.
Subcutaneous fat	3001	3441	2493	277	3357 [e]	2600 [f]	226	0.96
Mesenteric fat	936 [b]	1778 [a]	1135 [b]	138	1274	1293	113	0.08
Perirenal fat	857 [b]	1486 [a]	994 [b]	148	1121	1103	121	0.29

Values are least square means of main treatment effects. Number of animals = 8 for each mean under environment and 12 for each mean under diet effect.

[a, b] or [e, f] means in common row and within same treatment effect (environment or diet) and followed by different superscripts are significantly different ($p < 0.05$).

[z]CO = cold environment, COPF = cold pair-fed, and, WA = warm environment.

[y]CTL, LIPID are respectively control or lipid supplemented diet.

[x]E*D = Interaction of environment and diet.

SE = standard error of the least square means.

Table 3.6. Effect of environment or diet on total lipid composition (g / 100 g tissue) of *longissimus dorsi* muscle (LD), liver (LV), and, subcutaneous (SC), mesenteric (MS) and perirenal (PR) adipose tissues.

	Environment (E)[z]					Diet (D)[y]				E*D[x]
	CO	COPF	WA	SE	Prob.	CTL	LIPID	SE	Prob.	
LD	3.53	3.70	3.68	0.21	0.55	3.47	3.59	0.17	0.64	0.46
LV	5.75	5.96	5.78	0.16	0.31	5.66	5.87	0.14	0.30	0.96
SC	69.87	72.54	72.81	3.21	0.78	69.48	74.00	2.62	0.25	0.14
MS	82.90	84.57	84.60	2.53	0.86	85.09	82.96	2.07	0.48	0.21
PR	89.58	86.41	89.53	2.20	0.52	88.06	88.94	1.80	0.73	0.57

Values are least square means of main treatment effects. Number of observation per mean = 8 for environment and 12 for diet.

[z]CO = cold environment, COPF = cold pair-fed, and, WA = warm environment.

[y]CTL, LIPID = control and lipid supplemented diet respectively.

[x]E*D = Interaction of environment and diet.

SE = standard error of the least square means ($p > 0.05$).

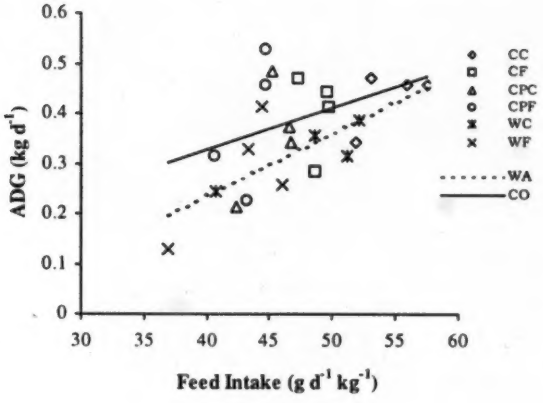

Figure 3.1. Relationship between average daily gain (ADG) and feed intake in sheep. Regression line and curve were derived from least squares regression equations are $Y = -0.009 + 0.008X$, $R^2 = 0.18$, $p = 0.10$ for cold environment (CO), and $Y = -0.264 + 0.012X$, $R^2 = 0.50$, $p = 0.05$ for warm environment (WA). CC = cold- control, CF-cold fat, CPC-cold pairfed control, CPF- cold pairfed fat, WC-warm control and WF- warm-fat treatments. Each point on the figure represents an individual animal. N = 4 animals per treatment.

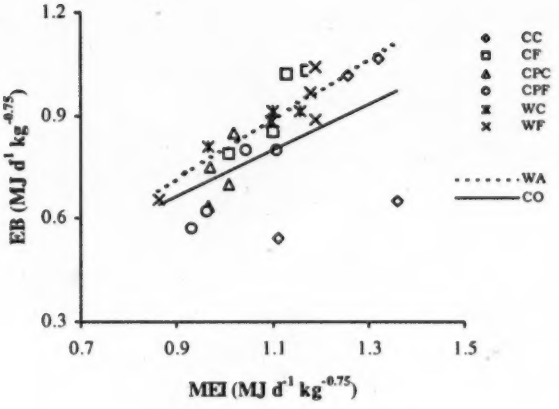

Figure 3.2. Relationship between energy balance (EB) and metabolizable energy intake (MEI) in sheep.

Regression lines were derived from least square regression and equations are $Y = 0.060 + 0.672X$, $R^2 = 0.27$, $p = 0.04$ for cold environment (CO), and $Y = -0.072 + 0.873X$, $R^2 = 0.84$, $p = 0.001$ for warm environment (WA). CC = cold- control, CF-cold fat, CPC-cold pairfed control, CPF- cold pairfed fat, WC-warm control and WF- warm-fat treatments. Each point on the figure represents an individual animal. N = 4 animals per treatment.

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4. Effect of Environmental Temperature and Dietary Lipid Supplement on Activity and Protein Abundance of Acetyl-CoA Carboxylase and Fatty Acid Synthase in Skeletal Muscle, Liver and Adipose Tissues of Sheep

4.1. INTRODUCTION

Acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS) control the rates of fatty acid synthesis in animal tissues. Two isoforms of ACC have been identified and described in the research literature. ACC-α (with a molecular weight of 265-kDa) is the rate limiting enzyme in the biogenesis of long chain fatty acids and ACC-β (with a molecular weight of 280-kDa) is believed to control mitochondrial fatty acid oxidation (Kim 1997). Fatty acid synthase may also be rate limiting in the long-term production of fatty acids in tissues in addition to the rate of delivery of acetyl-CoA subunits to the cytosolic sites of biosynthesis (Volpe and Vagelos 1976). However, little is known about the regulation of both ACC and FAS in ruminants.

In vitro studies have shown that the catalytic activity of ACC is very low compared to that of FAS in the synthesis of long chain fatty acids (Numa and Tanabe 1984). Acetyl-CoA carboxylase has also been found to have a wider tissue distribution than might be predicted by rates of lipogenesis (Thampy 1989; Bianchi et al. 1990; Iverson et al. 1990; Louis and Witters 1992). While the highest activity of ACC is found in traditional lipogenic tissues such as adipose tissue and mammary gland, the enzyme is also detectable in the heart and skeletal muscle tissues which have low rates of lipogenesis (Thampy 1989; Bianchi et al. 1990; Lopaschuk et al. 1994). With FAS there is evidence from studies in nonruminants that its synthesis is a regulated process that is

affected by diet (Clarke and Jump 1993), hormones and physiological status of the animal. The rates of transcription and the stability of its mRNA (Iritani et al. 1992; Hillgartner et al. 1995) control FAS levels. Both the activity of ACC and FAS and the rates of fatty acid synthesis in tissues fluctuate rapidly in response to various internal and external signals that affect lipogenesis, such as hormonal (Fukuda et al. 1992), dietary (Clarke and Jump 1996), developmental (Iritani et al. 1993), and genetic factors.

In Chapter three, it was shown that environmental temperature and dietary lipid supplement influenced the rates of fatty acid synthesis in adipose tissue of sheep. This tissue is the major site of synthesis of fatty acids in ruminant species and also expresses the lipogenic enzyme genes. Northern-blot analyses have revealed that the mRNA concentrations of each of the lipogenic enzymes of rat adipose tissue was the same as that of the liver (Katsurada et al. 1990), however, the catalytic activity of the enzyme in adipose tissues may actually be different from that in the liver.

The primary factor regulating fatty acid synthesis during cold exposure is likely to be the overall energy balance of the animal since the rates of lipid synthesis in tissues vary profoundly with nutritional and physiological status. Therefore, it is important that the lipogenic processes be regulated precisely in response to the ever-changing energy needs of the animals (Wakil et al. 1983). Generally, high carbohydrate, fat-free diets stimulate lipogenesis, at least in the liver of nonruminants, whereas dietary lipid has an inhibitory effect on adipose tissue lipogenic capacity in ruminants especially when the fat source is protected from rumen biohydrogenation (McNamara et al. 1995).

For this study, it was hypothesized that effects of thermal environment and dietary fat on fatty acid synthesis are due to alterations in the catalytic activity and protein

abundance of key enzymes regulating the fatty acid biosynthetic pathway. Therefore, the overall objective of the current study was to improve understanding of the mechanism regulating fatty acid synthesis in ruminants by studying the effect of environment and diet together with their interaction on key regulatory enzymes of the fatty acid biosynthetic pathway in wether lambs.

4.2. MATERIALS AND METHODS

4.2.1. Materials

[^{14}C]bicarbonate was purchased from Dupont, New England Nuclear (Boston, MA. U.S.A). Bovine serum albumin (essentially fatty acid free), polyethylene glycol (PEG) 8000, acetyl coenzyme A, malonyl coenzyme A and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Chemical Co (St. Louis, MO. U.S.A.). EcoLite™ liquid scintillation fluor was purchased from ICN (Costa Mesa, CA. U.S.A.). Hyper-Film ECL and Rainbow™ colored protein molecular weight markers were obtained from Amersham Life Science (Bukinghamshire, England). All other chemicals were purchased from Sigma or Fisher Scientific (Fair Lawn, NJ. U.S.A.), unless otherwise noted.

4.2.2. Animals

Animal management and treatments were as described in Chapter three. Briefly, a total of 24 Suffolk-Cross, 5 mo old wether lambs, were randomly divided into six treatment groups in a 3 X 2 factorial arrangement in a completely randomized design. The factors were three environments (+23-warm, 0 °C-cold, and, cold-pair-fed) and two dietary

regimens (control and lipid-supplemented). The experiment was divided into two periods with 12 animals used in each of the periods. All animals were shorn and treated with Ivomec antihelmintic (0.02 ml kg^{-1}) (MSD Agzet, PointeClaire, Dugorvale, Quebec), before being transferred into individual metabolism crates. The animals were maitanied in the metabolism crates throughout each period of study with weekly 4 h exercise. Animals were cared for according to guidelines of the Canadian Council on Animal Care (1993). Pair-fed animals in the cold environment were fed the level of feed consumed (per unit body weight) by animals in the +23 °C (warm) environment while all other animals were given *ad* libitum access to feed on either control or lipid supplemented barley-based diet containing 15% CP and 10.42 MJ estimated ME kg^{-1}.

After 5 wk on the temperature x diet treatments, a jugular venous catheter was inserted into each animal for radioisotope injection as described in Chapter three. Animals were killed 1 h after radioisotope injection using captive bolt stunning and rapid exsanguination. *Longissimus dorsi* muscle (between 12th and 13th rib), liver (left lobe) and adipose tissues (subcutaneous [between 12th and 13th rib], mesenteric [duodenal site] and perirenal [adjacent to left kidney]) were rapidly sampled within 10 min of stunning and snap frozen in liquid nitrogen. Frozen tissues were stored at –80 °C and were used for ACC and FAS activity assays, and western blotting analyses.

4.2.3. Enzyme Analyses

4.2.3.1. Sample Preparation

Frozen tissues were ground into powder under liquid nitrogen and 2 g were homogenized (teflon Potter-Elvehjem homogenizer) for 20 s in three times volume of buffer. The

buffer contained, 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, (pH 7.5 at 4 °C), 50 mM NaF, 0.25 M mannitol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 5 mM sodium pyrophosphate, 1 mM phenylmethanesulphonyl fluoride, 4 µg/ml soybean trypsin inhibitor, 4 µg/ml aprotinin, 4 µg/ml leupeptin, 4 µg/ml pepstatin A and 1 mM benzamidine. Homogenates were centrifuged (model J2-21, Beckman Instuments Inc., Irvine, CA) at 14,000 x g for 20 min at 4 °C and the supernatant used for extraction of ACC and FAS.

4.2.3.2. Acetyl-CoA Carboxylase Extraction and Assay

Polyethylene glycol-8000 (PEG) was added to 2 ml aliquots of the supernatant prepared from the above step to make a final concentration of 2 % PEG, stirred for 10 min at 4 °C and then centrifuged at 10,000 x g for 10 min in a JA-21 rotor. Acetyl-CoA carboxylase protein was precipitated from the supernate in 10 % PEG solution, agitated on ice for a further 10 min and centrifuged as before. The precipitate was collected and washed with 10% PEG 8000 in homogenizing buffer. After centrifugation (10,000 x g, 4 °C, 10 min), the pellet was resuspended in another buffer containing 100 mM Tris-HCl (pH 7.5 at 4 °C), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM Na pyrophosphate, 10 % (w/v) glycerol, 0.02 % sodium azide as preservatives, 4 µg/ml soybean trypsin inhibitor, 4 µg/ml aprotinin, 4 µg/ml leupeptin, 4 µg/ml pepstatin A, and 1 mM benzamidine. The protein content of the resuspended enzyme was determined by the bicinchoninic acid method according to the manufacturer's (Pierce, Rockford, IL) recommendations using bovine serum albumin as standard.

Acetyl-CoA carboxylase activity was assayed by the $H^{14}CO_3^-$ fixation method

(Thampy and Wakil 1985; Lopaschuk et al. 1994; Makinde et al. 1997). Final concentrations of assay components were 60.6 mM Tris-acetate, 2.12 mM ATP, 1.32 µM β-mercaptoethanol, 5.0 mM Mg acetate, 10 mM potassium citrate, 1.06 mM acetyl-CoA, 18.18 mM $NaHCO_3$, 0.33µCi/µmol $NaH^{14}CO_3$, 1 mg/ml fatty acid free-bovine serum albumin (BSA), pH 7.5. The reactions were started by addition of 10 µl of enzyme preparation (preincubated for 5 min) in a final assay mixture of 165 µl. After 4 min incubation at 37 °C, the reaction was stopped by addition of 25 µl of 10% perchloric acid. Reaction tubes were placed in a dessicator under vacuum to remove unreacted label and centrifuged at 3500 rpm (2900 x g) for 20 min with a Beckman J-6M/E centrifuge and a bucket rotor JS-5.2 (Beckman Instrument Inc., Irvine, CA). After centrifugation 160 µl of the supernate was transferred into a glass scintillation minivial and evaporated to dryness at 80 °C under gentle vacuum. The residue in the glass scintillation vial was dissolved in 100 µl H_20 and mixed with 4 ml scintillation fluid (EcoLite) for determination of radioactivity. Acetyl-CoA carboxylase activity was expressed as nmol of ^{14}C-bicarbonate incorporated into malonyl-CoA.min^{-1}.mg protein^{-1} (see Appendix 1 for calculations).

4.2.3.3. Fatty Acid Synthase Assay

Three-ml aliquots of the 14,000 x g supernatant from the sample preparation step were centrifuged in a Solvall® (Ultra *pro*80™, Du Pont Co. DE, USA) ultracentrifuge at 105,000 x g for 60 min at 4 °C to obtain adipose tissue, liver and muscle cytosol. After centrifugation, the supernatant was brought to saturation with saturated ammonium sulfate solution (containing 3 mM EDTA and 1 mM β-mercaptoethanol) and stirred for 60 min on ice. The precipitate was collected by centrifugation at 105,000 x g for 60 min.

The pellet was dissolved in 5 to 10% of the original volume of the homogenate buffer and was centrifuged briefly to remove insoluble protein and the supernatant used for FAS activity determination.

The protein content of the supernatant was determined using the bicinchoninic acid protein reagents (Pierce, Rockford, IL). Fatty acid synthase activity was assayed according to Nepokroeff et al. (1975) by measuring the malonyl-CoA and acetyl-CoA dependent oxidation of NADPH. The reaction was carried out at 30 °C using a Varian Cary UV-visible automated spectrophotometer equipped with a temperature controller for sample cuvettes (Varian Australia Pty Ltd. Mulgrave, Victoria, Australia). The slope of the assay at 340 nm was recorded by a computing integrator attached to the spectrophotometer, and the reaction was linear in the ranges employed. Final concentrations of reaction mixture were, (per ml), potassium phosphate buffer 500 µmol, EDTA 1µmol, β-mercaptoethanol 1 µmol, acetyl-CoA 35 nmol, malonyl-CoA 100 nmol and NADPH 100 nmol. Activity of FAS was calculated as nmol $NADPH.min^{-1}.mg\ protein^{-1}$ and data are presented as nmol palmitate $synthesized.min^{-1}.mg^{-1}$ enzyme protein by dividing the former (i.e. nmol $NADPH.min^{-1}.mg^{-1}$) by 14.

4.2.3.4. Western Blot Analyses of ACC and FAS

Subcutaneous or perirenal (20 µg), or of mesenteric (25 µg) adipose tissue protein, or 60 µg of muscle or liver protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). For each tissue, gels were run in duplicate and details of western blotting analysis are provided in Appendix 2. After electrophoresis on a 5% gel (2.5 h at 100 volts) using a Bio-Rad Mini-PROTEAN

II gel unit (Bio-Rad Laboratories, Missisauga, ON), the fractionated proteins were transferred in a Bio-Rad Mini Trans-Blot electrophoretic transfer system (Bio-Rad Laboratories, Missisauga, ON) to nitrocellulose membranes (Micron Separation Inc. Westborough, MA). For ACC, membranes were probed with peroxidase-conjugated-streptavidin (Jackson ImmunoResearch laboratories, Inc. WestGrove, PA). Fatty acid synthase was probed for with mouse anti-FAS monoclonal antibody and a secondary goat anti mouse-horse radish peroxidase (Chemicon International Inc. Temecula, CA). After washing the membranes, chemiluminescent detection was performed on them using an ECL™ western-blotting kit (Amersham Life Science, Buckinghamshire, England), followed by autoradiography.

4.2.4. Statistical Analyses

Data were analyzed as a 3 x 2 factorial design using the general linear model procedure of the Statistical Analysis System Institute (SAS 1996). The model consisted of period, environment (cold, cold-pair-fed and warm), diet, diet x environment, and, period x diet x environment effects. Interactions of environment or diet with period (that is, period x environment or period x diet) were pooled together with the standard error estimate. The least square means for period were tested against the standard error means square while environment, diet and environment x diet were tested using mean square for period x diet x environment as the error term. Where the period effect was not significant data were reanalyzed as a completely randomized design with model statement containing environment, diet, and environment x diet and tested against the standard error. In both models and where significant differences were obtained, comparisons of means were

made using the least significant difference procedure of SAS. All data are presented as least square means together with their pooled standard error. Probability of means less than .05 are considered to be significant.

4.3. RESULTS

4.3.1. Enzyme activity

Acetyl-CoA carboxylase activity measured in cytosolic extracts from frozen tissue samples is shown in Table 4.1. In lamb *longissimus dorsi* muscle, there was no effect of environmental temperature or lipid supplementation of diets of lambs on ACC activity. However, ACC activity was highest during warm exposure and lowest when animals were exposed to cold environmental temperature ($p > 0.05$), and there was a trend toward a temperature x diet interaction ($p = 0.06$). The interaction means (± SEM) for ACC activity in *longissimus dorsi* were 0.95 ± 0.14, 0.81 ± 0.01, 0.68 ± 0.09, 1.24 ± 0.14, 1.34 ± 0.45, and 0.90 ± 0.07 nmol $H^{14}CO_3$ per min per mg protein respectively for cold-control, cold-lipid, cold-pairfed-control, cold-pairfed-lipid, warm-control and warm-lipid treatment combinations. Statistical analysis of ACC activity data from the liver showed that the period effect was significant ($p = 0.04$). Therefore the model statement for Environment, Diet and Environment x Diet effects were tested using Period x Environment x Diet as an error term. Results showed that dietary lipid supplementation depressed ($p < 0.05$) ACC activity whereas cold exposure of the lambs during pair feeding enhanced ($p < 0.05$) ACC activity, so there was a significant temperature effect.

Activity of ACC was also determined in the three adipose tissue depots (subcutaneous, mesenteric and perirenal). Acetyl-CoA carboxylase activity in

subcutaneous and mesenteric adipose tissues was not significantly affected by exposure of the animals to a cold environment. Although in mesenteric adipose tissue, cold exposure reduced ($p > 0.05$) ACC activity by 30 %. Unlike in the liver, dietary fat supplementation increased ($p < 0.05$) ACC activity in both subcutaneous and mesenteric adipose tissue depots. There was no interaction between environmental temperature and diet on the measured activity of ACC except in mesenteric adipose tissue ($p = .074$) where the interaction approached significance. In this case, lipid supplementation tended to increase ACC activity in the warm and also in cold-pairfed animals but decreased the enzyme activity in the cold environment during *ad libitum* feeding.

In perirenal adipose tissue, both exposure of the animals to a cold environment and supplementing their diet with a lipid source increased the activity of ACC. Dietary fat increased ACC activity in this tissue by 40 % compared to control diet. The increase in ACC activity as a result of cold exposure was 20 – 30 % higher than in the warm environment. Notwithstanding the increase, the effects of environment and diet on ACC activity in perirenal fat were not significant ($p > 0.05$). In general, ACC activity was 20-fold higher in adipose tissues than in skeletal muscle and 10-fold higher than that observed in the liver.

Results of FAS activity in skeletal muscle (*longissimus dorsi*), liver and the three adipose tissue depots are presented in Table 4.2. Fatty acid synthase activity was not affected by environment or dietary lipid supplementation in either *longissimus dorsi* muscle or liver. In subcutaneous adipose tissue, cold exposure reduced ($p < 0.05$) FAS activity in *ad libitum* fed animals but not in the pair-fed groups. In mesenteric adipose tissue, cold exposure did increase FAS activity only in the cold-pairfed groups ($p < 0.05$).

There was also a slight increase in FAS activity in perirenal adipose tissue from animals exposed to a cold environment but this was not significant ($p > 0.05$). The effect of dietary lipid supplementation on all three adipose tissue depots was similar. Supplementing the diets of the wether lambs with a lipid source significantly reduced ($p < 0.05$) the cytosolic activity of FAS by 30 – 50 %.

4.3.2. SDS/PAGE and Western-Blot Analyses

SDS/PAGE and western-blots of ACC and FAS demonstrated similar molecular-mass for these enzymes among the skeletal muscle, liver and adipose tissues. However, skeletal muscle of sheep also contained another isoform of ACC of higher molecular mass, which was not present in liver or in adipose tissue. Furthermore, the results of western-blot analyses demonstrated that the two bands observed in *longissimus dorsi* blots corresponded to two isoforms of ACC with molecular weights of 280 and 265 kDa (Figure 4.1-A). The 280-kDa subtype was the more predominant isoform. The arbitrary densitometry units for immunodetectable ACC protein in *longissimus dorsi* muscle are presented in Table 4.3. Cold exposure of animals in this study increased ($p < 0.05$) the abundance of the ACC-280, and ACC-265 isoform also increased slightly in the cold compared to the warm environment, but this was not significant ($p > 0.05$). Although slight decreases in enzyme protein concentration were observed, there was no significant effect of dietary lipid supplementation on the expression of the two isoforms of ACC in skeletal muscle.

The liver and adipose tissue revealed the presence of only one isoform of ACC corresponding to 265-kDa MW as shown in Figures 4.1-B, and 4.2-A to C. As presented

in Table 4.3, neither environment ($p = 0.56$) nor diet ($p = 0.83$) influenced ACC protein abundance in the liver. Higher levels of ACC protein abundance were observed in all three adipose tissue depots in response to cold exposure. There was no significant effect of environmental temperature on ACC protein abundance in subcutaneous adipose tissue. In mesenteric adipose tissue, cold exposure increased ($p < 0.05$) ACC protein abundance by more than 90 % (including the pair-fed groups) compared to the warm environment.

The trend in perirenal adipose tissue was similar to that observed in mesenteric adipose tissue with over 60 % higher ACC protein abundance in the cold compared to the warm environment, but this was not statistically significant. When the diets of the wethers were supplemented with a lipid source, ACC protein abundance in subcutaneous adipose tissue was significantly depressed ($p < 0.05$). In both mesenteric and perirenal adipose tissues there were small depressions in ACC protein expression which were not significant ($p > 0.05$). There were no significant interactions of environment and diet on ACC protein abundance in any of the tissues in this study.

Fatty acid synthase protein abundance was detected using mouse anti-FAS monoclonal antibody and a secondary goat anti mouse-horse radish peroxidase. The results are shown in Table 4.4 and Figures 4.3 and 4.4. Western-blots indicated the presence of only one species of FAS with a molecular weight corresponding to 260-kDa in all tissues. In skeletal muscle, FAS protein abundance was slightly but not significantly increased by exposure of the animals to a cold environment. In liver, cold exposure of the pair-fed animals increased ($p < 0.05$) FAS protein abundance. In adipose tissue, cold exposure significantly increased ($p < 0.05$) FAS protein abundance in subcutaneous, mesenteric and perirenal adipose tissue.

Dietary lipid slightly reduced FAS protein abundance in *longissimus dorsi* but this was not significant. There was no significant effect of dietary lipid on FAS protein abundance in liver. Dietary lipid supplementation however, slightly lowered FAS protein expression in all the three adipose tissue depots but the effects were small and not significant. There were no interactions between the environment and diet on FAS protein expression in skeletal muscle, liver and adipose tissues of sheep in this study.

4.4. DISCUSSION

Despite the central role played by the lipogenic pathway in energy homeostasis and its unique role in certain specialized tissues, little is known about the regulation of the enzymes of this pathway in response to varying environmental temperatures and diet in ruminants. In Chapter two, it was demonstrated that environmental temperature had regulatory effects on activity and protein expression of ACC and FAS in adipose tissue of sheep. The study also indicated that feed restriction depressed the activities of these two enzymes in subcutaneous and mesenteric adipose tissues from sheep. Furthermore, in Chapter three, exposure of sheep to two environmental temperatures and supplementing their diet with a lipid source had significant effects on rates of fatty acid synthesis. Therefore, in the current study, the activity and protein expression of the key regulatory enzymes ACC and FAS were studied further as indicators of fatty acid synthesis in sheep, since their positive relationship to lipogenesis in pig adipose tissue has been previously demonstrated (O'Hea and Leveille 1969).

In skeletal muscle, the lack of a significant effect of temperature or diet on ACC activity, is in agreement with reports of Winder et al. (1995) who showed that ACC

activity in quadriceps muscle of rat was not significantly reduced by fasting and refeeding, whereas liver ACC activity was significantly reduced. In this study, the liver appears to play a minor role in sheep fatty acid synthesis as demonstrated by the lower enzyme activity, protein abundance and rates of fatty acid synthesis (data in Chapter three) when compared to adipose tissue. The activities of ACC and FAS were at least 10 times lower in the liver than in adipose tissue. Similarly, protein abundances of ACC and FAS were lower in the liver compared to adipose tissues, and this reflected the lower rates of fatty acid synthesis reported in Chapter three. These results are in general agreement with Hood et al. (1980) who reported that *de novo* fatty acid synthesis rate was very low in ruminant liver.

The pattern of ACC activity during exposure of sheep to the two environmental temperatures was not the same in all three adipose tissue depots studied, as cold exposure decreased enzyme activity in subcutaneous and mesenteric but elevated enzyme activity in perirenal adipose tissue. The increased activity of ACC in perirenal adipose tissue of sheep during exposure to a cold environment was in agreement with Nicholls and Locke (1983) who showed that during long term exposure to a cold environment when fatty acid oxidation was enhanced, ^{3}H incorporation into fatty acids was also increased. Buckley and Rath (1987) also showed that increased rates of fatty acid synthesis in the cold environment were a consequence of increased activities of ACC and FAS resulting from increased enzyme protein expression.

The effects of dietary lipid supplementation of sheep on ACC activity in adipose tissue were more consistent compared to the effects of environment. However, unlike the commonly reported decrease in lipogenic enzyme activity in response to dietary fat, ACC

activity in adipose tissue of sheep was increased. At the same time, decreased rates of *in vivo* fatty acid synthesis were measured in the adipose tissue (Chapter three, Table 3.3). Only in perirenal adipose tissue did an increase or a decrease in measured enzyme activity as a result of cold exposure or dietary fat supplementation reflect a respective increase or decrease in the amount of enzyme protein abundance. However, when all the treatments were pooled in a regression analysis so as to describe a general pattern of relationship, both activity and protein abundance were either not related or were negatively related (Figures 4.5 and 4.6). The reasons for this type of negative relationship between ACC activity and protein abundance in adipose tissue are not clear. It is possible that differences in the rates of recruitment of precursors of fatty acid synthesis by the tissues or in post-translational modification of the enzyme (Cousin et al. 1993) following adaptation to different environmental temperatures could be implicated. Thus, protein abundance was not a good indicator of enzyme catalytic activity in the current study.

Acetyl-CoA carboxylase has been shown to be widely distributed in a number of different mammalian tissues (Thampy 1989; Iverson et al. 1990; Trumble et al. 1991, 1995). From the present study, two isoforms of ACC (ACC-265 and ACC-280) were identified in skeletal muscle and one both in liver and adipose tissue by binding of streptavidin to the biotin moiety of the enzyme. Although ACC-265 and ACC-280 both contain a covalently bound biotin prosthetic group, the former is immunologically distinct from the latter (Thampy 1989; Bianchi et al. 1990; Witters et al. 1994). The ACC isozymes have also been shown to differ in their kinetics properties. ACC-280 has increased citrate dependence (higher K_a) and a twofold higher K_m for acetyl-CoA (Bianchi et al. 1990, 1992) compared to ACC-265. Presently, the characteristics and

regulation of ACC-265 are better understood than ACC-280. ACC-265 has been shown to be regulated by both long- and short-term control mechanisms (Kim et al. 1989). In the current study, the 265-kDa isoform of ACC was demonstrated in adipose tissue and liver.

In *longissimus dorsi* muscle, there was no effect of cold exposure or dietary fat on the two isoforms, however, the relative expression of the different isoforms was different with the 280-kDa being more abundant than the 265-kDa isoform. The physiological significance of ACC-280 in skeletal muscle of sheep at the present time is not known. However, the association of ACC-280 with cellular membranes (mitochondria, nuclear, and endoplasmic) may, reflect its function within the cell (Abu-Elheiga et al. 1997). The presence of the 280-kDa isoform in nonlipogenic tissues such as heart (Thampy 1989; Lopaschuk et al. 1994) and skeletal muscle in which very little fatty acid synthesis occurs may indicate its importance in the regulation of mitochondrial fatty acid oxidation (Lopaschuk et al. 1994). In addition, the 280-kDa isoform found in skeletal muscle may be important in the provision of malonyl-CoA for elongation of fatty acids into very long chain fatty acids that are required for the structure of cellular membranes (Abu-Elheiga et al. 1997). The higher abundance of ACC-280 protein compared to ACC-265 in *longissimus dorsi* muscle from sheep therefore may be an important control point for regulating energy metabolism of the animals by regulating oxidation of fatty acids during cold exposure when energy demands are high.

The functions of the 265-kDa isoform in muscle are not known but may not be different from the function performed by the same isoform in the liver and adipose tissue. Unlike the 265-kDa isoform in adipose tissue, the 265-kDa isoform in the muscle was not significantly affected by the treatments imposed on the animals. The coexistence of the

two isoforms of ACC in the skeletal muscle of sheep will probably increase the complexity of understanding the regulation of this enzyme and its contribution to fat synthesis and deposition in sheep.

Fatty acid synthase activity was increased in adipose tissue from cold exposed animals compared to that from animals housed in a warm environment. The increases in FAS activity and protein abundance were consistent with the increases observed in the rates of *in vivo* and *in vitro* fatty acid synthesis in adipose tissue as presented in Chapter three. The fact that FAS activity and protein abundance varied in a corresponding manner in relation to the rates of fatty acid synthesis suggests that changes in FAS activity or protein abundance in adipose tissue may both be used as indicators of rates of fatty acid synthesis in sheep. As shown in Figures 4.7 and 4.8, the relationships between FAS activity and protein abundance were linear but not significant.

In skeletal muscle (Figure 4.7-A), increases in protein expression were not related to changes in FAS activity, and in subcutaneous adipose tissue, the relationship between FAS activity and immunodetectable protein was negative (Figure 4.8-A). The liver (Figure 4.7-B), mesenteric (Figure 4.8-B) and perirenal (Figure 4.8-C) adipose tissues demonstrated increases in FAS activity with an increase in protein expression although the relationship was only significant for perirenal fat.

Decreased FAS activity in tissues from sheep following dietary lipid supplementation is in agreement with earlier observations in rats (Toussant et al. 1981), and with studies in sheep (Vernon 1976) that showed that increasing the lipid content of the diet from 4 to 8% (w/w) with protected tallow resulted in decreased rates of fatty acid synthesis and activities of several lipogenic enzymes in perirenal adipose tissue.

Fatty acid synthase proteins are thought to be regulated primarily by changes in its gene expression. The decreased FAS protein abundance following dietary lipid supplementation ($p > 0.05$) in the present study is in agreement with Blake and Clarke (1990) who reported that dietary fats decreased FAS gene expression in tissues of male Sprague-Dawley rats. Fatty acid synthase activity and protein abundance in adipose tissue also agreed with data presented in chapter three on reduced rates of fatty acid synthesis caused by dietary lipid supplementation. In suckling rats weaned to a high-fat diet, increases of FAS and ACC mRNA abundances and of activities in liver were prevented whereas weaning to high carbohydrate diet resulted in increased mRNA abundance and activities (Perdereau et al. 1990) of both enzymes. The current data are also in agreement with the results of Clarke and Jump (1994, 1996) who showed that dietary fat was a highly effective inhibitor of the expression of lipogenic enzymes and that part of the inhibition was due to the direct effects on the activity or concentration of the lipogenic enzymes.

The components of dietary fat, which may inhibit fatty acid synthesis, are most likely polyunsaturated fatty acids (PUFA) (Wilson et al. 1990). The intracellular pathway involved in regulation of lipogenic enzymes by PUFA has not been elucidated, however there is evidence that the inhibition of transcription by PUFA occurs by direct binding to a transcriptional factor that functions as a dominant repressor (Jump et al. 1993) coding for lipogenic enzymes.

Although the activity of ACC can be increased by allosteric factors and dephosphorylation as well as by increased gene expression (Hillgartner et al. 1995), increased FAS activity has been attributed mainly to increased gene expression. The

changes in the FAS activity in the current study were reflected by slight alterations in the amount of enzyme protein. This relative change in immunodetectable enzyme protein content may be governed by the rate of synthesis and stability of its mRNA in a tissue specific manner (Iritani et al. 1992; Clarke 1993; Hillgartner et al. 1995).

Changes in FAS activity and protein abundance in the current study were positively related in the liver, mesenteric and perirenal adipose tissue (Figures 4.7-B and 4.8-B and C). Fatty acid synthase activity and protein expression both showed an upward trend during cold exposure whereas a downward trend was seen with lipid supplementation. It is not known what other factors may be involved in the elevation of FAS in the cold but differences in intakes of animals, the overall energy balance, and subtle changes in insulin and T_3 status over the period of the experiment may be implicated. Katsurada et al. (1990) suggested that insulin stimulated the transcription of the FAS gene, whereas posttranscriptional regulation of FAS synthesis was accomplished by alterations of mRNA stability. Because plasma insulin concentration increased significantly with time moreso in the cold in this study, it is possible that these hormonal changes played some role in the upregulation of FAS protein abundance measured in tissues of the cold-exposed animals.

Recently, Hsu et al. (1996) provided evidence for the existence of two promoters (PI and PII) implicated in regulating human FAS and suggested that promoter II activity was probably needed for low-level constitutive expression. Under lipogenic conditions, promoter II activity is minimized to remove a transcription block in favor of efficient transcription of promoter I. It is not known at present whether ruminant FAS is regulated by two promoters or whether multiple promoters will translate into multiple isozymes of

the FAS enzyme. However, the current study showed that skeletal muscle, liver, and adipose tissues of sheep all contained a single form of FAS protein with a molecular weight corresponding to 260-kDa.

Feeding a barley-based diet, as in the present study, provides increased levels of glycolytic intermediates to maintain the stability of the lipogenic enzyme mRNAs (Goodridge 1987; Katsurada et al. 1990) and this may have helped to increase the enzyme activity along with increases in ACC and FAS protein expression reported for the animals in the cold. Buchanan-Smith et al. (1973) showed that feeding a high carbohydrate diet may increase the activities of lipogenic enzymes due to increased energy intake and increased plasma glucose or glucose precursors. In a ruminant animal, glucose may not be absorbed in substantial quantities from the gastrointestinal tract except when high starch diets are fed. The supply of propionate will likely also be increased as a result of increased concentrate and starch feeding. As reported in Chapter three, there was an increase in post-prandial plasma insulin concentration after feeding the carbohydrate rich diet and the insulin levels also increased weekly. The sustained increase in plasma insulin level could have promoted high activities and protein expression of ACC and FAS. The change in activities of ACC and FAS in response to lipid supplementation tended to be in opposite directions in this study, suggesting that they respond differently to dietary signals.

The ability of insulin to alter the catalytic properties of ACC under different physiological conditions may occur through a variety of mechanisms including dephosphorylation of serine residues in the protein (Witters et al. 1988), which will result in enzyme activation, direct phosphorylation at an insulin site (Borthwick et al. 1990) or

possibly through the action of a molecular effector (Haystead and Hardie 1986). Therefore, it may not be surprising to see that ACC activity increased when at the same time FAS was decreased since ACC activity is regulated by posttranslational changes in phosphorylation state whereas, FAS is more subject to changes in gene expression. However, since most of the studies on posttranslational modification of ACC have been conducted on adipose tissue from rats, it is not certain whether all these mechanisms are necessarily applicable to ruminant animals.

In conclusion, environmental temperature and dietary lipid affected both ACC and FAS activity and protein expression but the response of the two enzymes was different. The effect of environmental temperature was more pronounced on FAS compared to ACC. The effect of dietary lipid was generally significant in both ACC and FAS but resulting, respectively, in increases and decreases in enzyme activity. In adipose tissues, increases in ACC activity may not be due to an increase in the amount of enzyme protein since no relationship was established between activity and protein abundance. With FAS however, increased activity was probably brought about by an increase in enzyme protein content particularly in liver, mesenteric and perirenal adipose tissue. The increases in FAS activity were related to similar increases in the rates of fatty acid synthesis presented in Chapter three. This may indicate that the absolute increment in enzyme activity or protein abundance in adipose tissue from cold exposed animals was to support an enhanced rate of fatty acid synthesis or an increased fatty acid turnover.

Table 4.1. Effects of environmental temperature and dietary lipid supplement on activity of acetyl-CoA carboxylase in skeletal muscle, liver and adipose tissues from sheep.

	Environment (E)[z]				Diet (D)[y]			E*D
Tissue	CO	COPF	WA	SE	CTL	LIPID	SE	Pr >
Longissimus dorsi	0.88	0.96	1.12	0.14	0.99	0.98	0.12	0.06
Liver	2.68 [b]	3.05 [a]	2.43 [b]	0.32	2.94 [e]	2.50 [f]	0.26	0.18
Subcutaneous fat	18.70	18.81	20.47	2.57	16.38 [f]	22.27 [e]	2.10	0.15
Mesenteric fat	23.53	25.59	35.34	4.55	22.90 [f]	32.76 [e]	3.60	0.07
Perirenal fat	29.40	26.94	22.23	5.00	21.95	30.42	4.10	0.34

ACC activity was determined as described under 'Materials and Methods' and expressed as nmol $H^{14}CO_3$ incorporated into acid stable malonyl-CoA.min^{-1}.mg $protein^{-1}$. Results are presented to show main treatment effects (environment n = 8; diet n = 12) since the interactions of environment and diet (E*D) were not significant.

[z]CO = cold environment; COPF = cold-pairfed; WA = warm environment.

[y]CTL = control diet; LIPID = lipid-supplemented diet.

SE = standard error of least square means.

[a, b] or [e, f]: Means within rows with different superscripts and under same treatment effect are significantly different, $p < 0.05$.

Table 4.2. The effect of cold temperature exposure and lipid supplementation of ruminant diet on fatty acid synthase activity in skeletal muscle, liver and adipose tissues.

	Environment (E)[z]				Diet (D)[y]			E*D
Tissue	CO	COPF	WA	SE	CTL	LIPID	SE	Pr >
Longissimus dorsi	0.74	0.77	0.69	0.22	0.80	0.67	0.18	0.39
Liver	1.00	0.70	0.92	0.18	0.98	0.77	0.14	0.70
Subcutaneous fat	12.44 [b]	19.17 [a]	18.47 [a]	2.00	19.74 [e]	13.65 [f]	1.64	0.33
Mesenteric fat	14.65 [b]	21.52 [a]	14.74 [b]	2.20	19.84 [e]	14.11 [f]	1.81	0.11
Perirenal fat	17.61	20.72	15.44	3.04	23.76 [e]	12.09 [f]	2.48	0.84

FAS activity was determined as described under 'Materials and Methods' and expressed as nmol palmitate synthesized.min^{-1}.mg $protein^{-1}$. Only the main treatment effects are shown since the interactions of temperature x diet (E*D) were not significant. For temperature effect, n = 8; for diet effect n = 12.

[z]CO = cold environment; COPF = cold-pairfed; WA = warm environment.

[y]CTL = control diet; LIPID = lipid-supplemented diet.

SE standard error of least square means.

[a, b] or [e, f] Means within rows with different superscripts and under same treatment effect are significantly different, $p < 0.05$.

Table 4.3. Effect of environmental temperature and dietary lipid supplement on ACC protein abundance in skeletal muscle, liver and adipose tissues from sheep.

	Environment (E)[z]				Diet (D)[y]			E*D[x]
Tissue[w]	CO	COPF	WA	SE	CTL	LIPID	SE	Pr >
LD-280	6.45 [a]	6.20 [a]	5.05 [b]	0.38	6.02	5.79	0.31	0.93
LD-265	2.70	2.68	2.09	0.35	2.65	2.33	0.28	0.99
Liver	2.72	2.76	2.02	0.54	2.43	2.57	0.44	0.97
Subcutaneous fat	12.89	11.12	11.44	1.37	13.47 [e]	10.16 [f]	1.12	0.68
Mesenteric fat	10.23 [a]	8.06 [a]	4.77 [b]	0.76	8.13	7.24	0.62	0.69
Perirenal fat	3.10	3.18	1.94	0.63	3.32	2.17	0.51	0.90

Values are least square means of densitometry units / amounts of cytosolic protein loaded. Number of observation per mean = 8 for environment and 12 for diet.

[z]CO and WA = cold and warm environments respectively. COPF = Animals in the cold environment but pair-fed to the intake of those in the warm.

[y]Control (CTL) barley based diet or with lipid (LIPID) supplementation.

[x]E*D = Environment x diet interaction

[w]LD-280/265 = *longissimus dorsi* muscle, with 280-kDa or 265-kDa isoform. Liver, and subcutaneous, mesenteric and perirenal fat all contained the 265-kDa isoform of ACC.

SE = Standard error of least square means for treatment effects.

[a,b] or [e, f] Values in rows followed by different superscripts within each treatment group (temperature or diet) are significantly different ($p < 0.05$).

Table 4.4. Effect of environmental temperature and dietary lipid supplement on FAS protein abundance in *longissimus dorsi* muscle, liver and adipose tissues from sheep.

	Environment (E)[z]				Diet (D)[y]			E*D[x]
Tissue	CO	COPF	WA	SE	CTL	LIPID	SE	Pr >
Longissimus dorsi	3.51	4.11	2.12	.92	3.54	2.95	0.75	0.96
Liver	11.70 [ab]	12.61 [a]	8.77 [b]	1.28	10.66	11.40	1.04	0.76
Subcutaneous fat	15.69 [a]	12.09 [b]	11.98 [b]	0.93	13.99	12.51	0.76	0.53
Mesenteric fat	11.77 [a]	11.27 [ab]	8.48 [b]	1.07	11.16	9.86	0.87	0.39
Perirenal fat	17.03 [a]	18.76 [a]	14.21 [b]	1.20	17.77	15.56	0.98	0.66

Values are least square means of densitometry units. Number of observation per mean = 8 for environment and 12 for diet.

[z]CO and WA = cold and warm environments respectively. COPF = Animals in the cold environment but pair-fed to the intake of those in the warm

[y]Control (CTL) barley based diet or with lipid (LIPID) supplementation.

[x]E*D = Environment x diet interaction

SE = Standard error of least square means for main treatment effects.

[a,b] Values in common row within temperature effect and followed by different supercripts are significantly different ($p < 0.05$)

Figure 4.1. Results of western-blot analyses of ACC protein expression in *longissimus dorsi* muscle (panel A) and liver (panel B) from sheep exposed to different temperature treatments and receiving a barley-based diet with or without a lipid-supplement.
Western-blot and SDS-PAGE were performed as described under 'Materials and Methods' by loading 60 μg protein into each lane and transferred to a nitrocellulose membrane. After blocking the membranes with 10 % skim milk they were incubated with peroxidase-labelled streptavidin. Lanes 1 &2, 3 & 4, 5 & 6, 7 & 8, 9 & 10, and 11 & 12 respectively represent samples from animals on warm-control, warm-fat, cold-control, cold-fat, pairfed-cold-control, and pairfed-cold-fat of environment by diet treatment combinations.

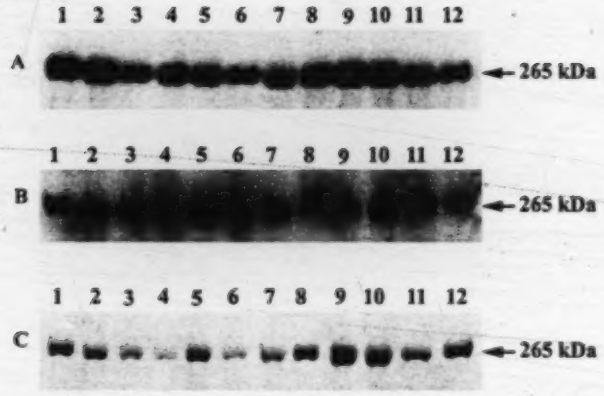


Figure 4.2. The effects of cold environmental exposure and a dietary lipid supplement on ACC protein abundance in adipose tissues from wether lambs fed a barley based diet. Panels A, B and C are representative of subcutaneous, mesenteric and perirenal adipose tissues respectively. Lanes 1 &2, 3 & 4, 5 & 6, 7 & 8, 9 & 10, and 11 & 12 respectively represent samples from animals on warm-control, warm-fat, cold-control, cold-fat, pairfed-cold-control, and pairfed-cold-fat of environment by diet treatment combinations. 20 μg of subcutaneous or perirenal, or 25 μg of mesenteric adipose tissue total protein were applied to each lane. ACC was detected using peroxidase-conjugated streptavidin as described under Materials and Methods.

Figure 4.3. The effect of environmental temperature and a dietary lipid supplement on FAS protein abundance in skeletal muscle (panel A) and liver (panel B) from wether lambs fed a barley-based diet.

SDS-PAGE was carried out by loading a 60 μg protein sample into each lane. Blots were probed for FAS using mouse anti-FAS monoclonal antibody and a secondary goat anti-mouse horseradish peroxidase. Lanes 1 &2, 3 & 4, 5 & 6, 7 & 8, 9 & 10, and 11 & 12 respectively represent samples from animals on warm-control, warm-fat, cold-control, cold-fat, pairfed-cold-control, and pairfed-cold-fat of environment by diet treatment combinations. Bands shown correspond to 260,000 Da.

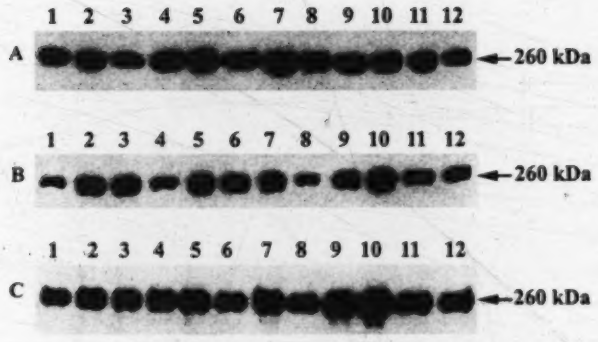


Figure 4.4. The effects of cold environmental exposure and a dietary lipid supplement on FAS protein expression in adipose tissues of wether lambs fed a barley-based diet.

Panels A, B and C are representative of subcutaneous mesenteric and perirenal adipose tissues respectively. Lanes 1 &2, 3 & 4, 5 & 6, 7 & 8, 9 & 10, and 11 & 12 respectively represent samples from animals on warm-control, warm-fat, cold-control, cold-fat, pairfed-cold-control, and pairfed-cold-fat of environment by diet treatment combinations. 20 μg of subcutaneous or perirenal, or 25 μg of mesenteric fat total protein were applied to each lane and FAS detected as described for Fig. 4.3. Bands shown correspond to 260,000 Da.

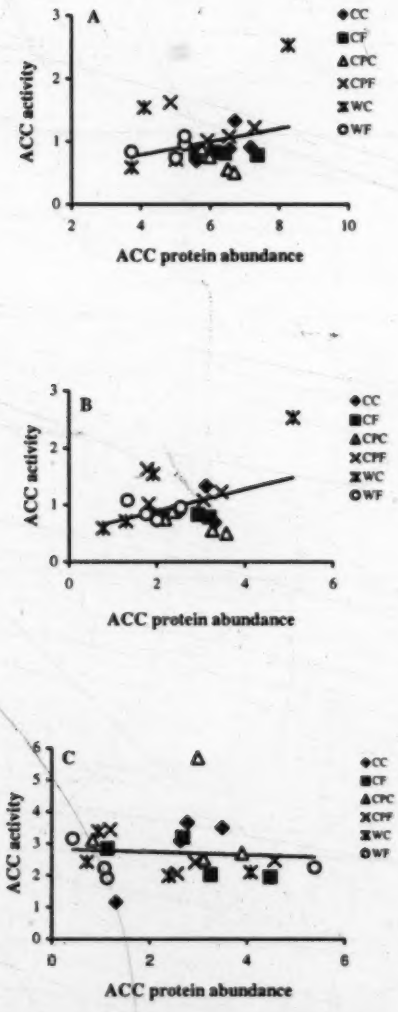
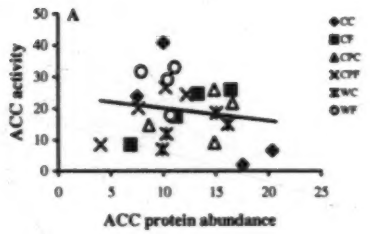
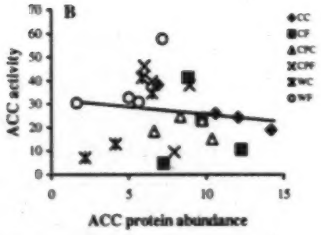


Figure 4.5. Relationship between acetyl-CoA carboxylase activity and immunodetectable protein in skeletal muscle (A, 280-kDa; B, 265-kDa) and liver (C).

Activity and protein abundance were determined as described under Materials and Methods. ACC activity was in nmol $H^{14}CO_3.min^{-1}.mg\ protein^{-1}$. Protein abundance was given as arbitrary densitometry units. Regression lines were generated from least squares and equations are: $Y = 0.37 + 0.10X$, $R^2 = 0.08$ (A); $Y = 0.51 + 0.19X$, R^2 0.17 (B) and $Y = 2.83 - 0.05X$, $R^2 = 0.01$ (C). CC = cold control, CF = cold-fat, CPC = cold-pairfed control, CPF = cold-pairfed fat, WC = warm-control and WF = warm-fat.





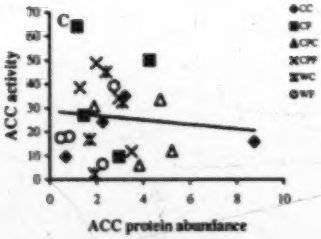


Figure 4.6. Relationship between acetyl-CoA carboxylase activity and immunodetectable protein in subcutaneous (A), mesenteric (B) and perirenal (C) adipose tissues.

Activity and protein abundance were determined as described under Materials and Methods. ACC activity was in nmol $H^{14}CO_3 min^{-1}$.mg $protein^{-1}$. Protein abundance was given as arbitrary units. Regression lines were generated from least squares and equations are: $Y = 24.07 - 0.40X$, $R^2 = 0.03$ (A): $Y = 32.16 - 0.66X$, $R^2 = 0.02$ (B) and $Y = 28.79 - 0.95X$, $R^2 = 0.01$ (C). CC = cold control, CF = cold-fat, CPC = cold-pairfed control, CPF = cold-pairfed fat, WC = warm-control and WF = warm-fat.

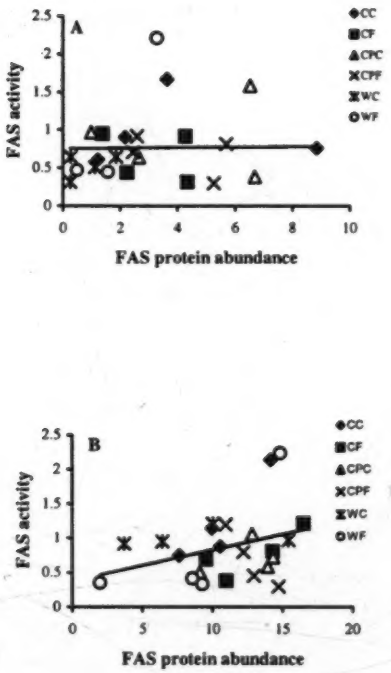


Figure 4.7. Relationship between fatty acid synthase activity and protein abundance in skeletal muscle (A) and liver (B).

Activity and protein abundance were determined as described under Materials and Methods. FAS activity was in nmol palmitate.min^{-1}.mg protein^{-1}. Protein abundance was given as arbitrary units. Regression lines were generated from least squares and equations are: $Y = 0.75 + 0.003X$, $R^2 = 0.0003$ (A) and $Y = 0.38 + 0.05X$, $R^2 = 0.11$ (B). CC = cold control, CF = cold-fat, CPC = cold-pairfed control, CPF = cold-pairfed fat, WC = warm-control and WF = warm-fat.

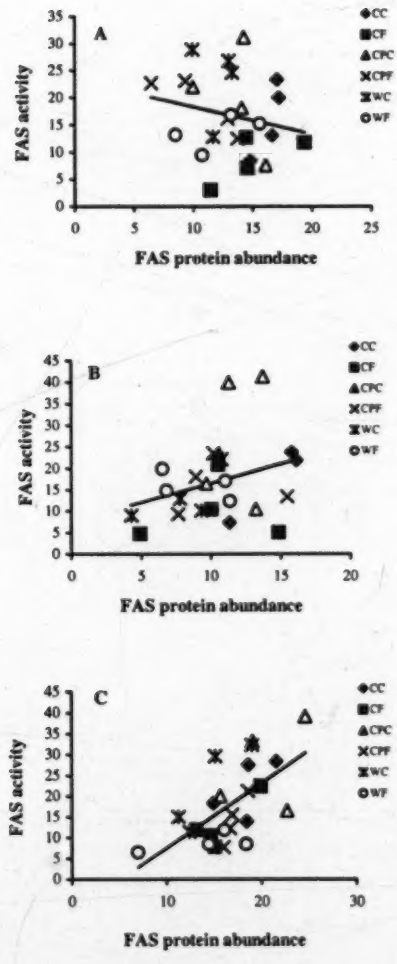


Figure 4.8. Relation between fatty acid synthase activity and protein abundance in subcutaneous (A), mesenteric (B) and perirenal (C) adipose tissues.

Activity and protein abundance were determined as described under materials and methods. FAS activity was in nmol palmitate.min^{-1}.mg protein^{-1}. Protein abundance was given as arbitrary units. Regression lines were generated from least squares and equations are: Y = 23.24 – 0.49, R^2 = 0.04 (A); Y = 7.54 + 0.90X, R^2 = 0.10 (B) and Y = - 8.01 + 1.56X, R^2 0.40 (C). CC = cold control, CF = cold-fat, CPC = cold-pairfed control, CPF = cold-pairfed fat, WC = warm-control and WF = warm-fat.

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5. Effects of β-Adrenergic Agonist L-644,969 on the Impact of Thermal Environment on *In Vitro* Fatty Acid Synthesis and Lipogenic Enzymes in Sheep

5.1. INTRODUCTION

Fatty acid synthesis in both white and brown adipose tissues in rats is stimulated *in vivo* by insulin and insulin is also important for promoting fat deposition in ruminants (Vernon et al. 1981; de la Hoz and Vernon 1993). The mechanism in rats involves parallel increases in the proportion of acetyl-CoA carboxylase (ACC) in its active form (McCormack and Denton 1977). In contrast, catecholamines have been shown to inhibit fatty acid synthesis in adipose tissue, and the mechanism is thought to involve beta-adrenoceptor (Shimazu and Takahashi 1980; Gibbins et al. 1985) or α-adrenergic receptor (Ly and Kim 1981) mediated inactivation of ACC activity.

The endogenous physiological β-adrenergic receptor (β-AR) agonists are norepinephrine and epinephrine. Oral administration of some synthetic β-AR agonists have been shown to cause modification of growth with increased accretion of skeletal muscle and decreased accretion of fat in cattle and sheep (MacRae et al. 1988). Three β-AR subtypes (β1-AR, β2-AR and β3-AR) have been described in the literature. The β-AR subtypes are present on most mammalian cells but the distribution of the subtypes and proportion of each varies between tissues in a given species (Mersmann 1998).

L-644,969 (Figure 5.1.) is a β-AR agonist that was developed for use as a leanness-enhancing agent and it is the stereoisomerically pure R,R isomer of 6-amino-alpha{[(1-methyl-3-phenylpropyl)amino]methyl}-3-pyridine methanol dichloride (Zhang and Grieve 1995). It has been shown that carcass fat content was significantly reduced

when L-644,969 is fed to rats at a diet concentration of 4 or 8 ppm (Zhang and Grieve 1995). There is also a linear reduction in the proportion of mammary, subcutaneous and perirenal fat depots with increased β-agonist administration. This showed that the β-agonist L-644,969 is effective in reducing fat deposition in the major fat depots. However, the exact metabolic mechanisms by which these β-AR agonists exert their effects are not fully understood, but may involve an indirect effect on the enzymes that regulate the pathway of fatty acid biosynthesis.

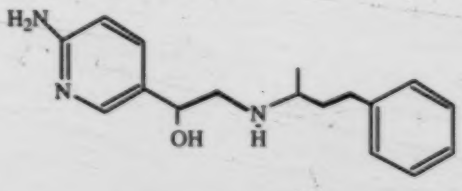

Figure 5.1. Structure of β-adrenergic agonist, L-644,969.

Tissue responses to adrenergic agents may be modified by the environment in which an animal is placed. In a cold environment, the activities of the sympatho-adreno-medullary system are stimulated, leading to increased release of endogenous adrenergic agonists (Sasaki and Weekes 1984) which may modify β-AR. The combined effects of environmental temperature exposure and β-AR agonist treatment on regulation of fat deposition in sheep are not known. In view of the importance of lipid as a body energy reserve and substrate supply to support metabolism in times of stress, it seemed important to quantify fatty acid synthesis and enzyme activity in sheep in different environments. Conducting these studies in the presence and absence of a β-AR agonist was expected to improve understanding of the regulation of lipid synthesis in sheep. Therefore, the aim of

the present studies was to determine the effect of L-644,969 on fatty acid synthesis in white adipose tissue from sheep exposed to two environmental temperatures. In addition, changes in activities and protein expression of the key regulatory enzymes, ACC and FAS, of the fatty acid biosynthetic pathway in skeletal muscles and adipose tissue of sheep were also investigated.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO USA) or Fisher Scientific (Fair Lawn, NJ. U.S.A.). Radiochemicals were obtained from Dupont, NEN (Boston, MA. U.S.A). EcoLite liquid scintillation fluor was obtained from ICN (Costa Mesa, CA). Hyper-Film ECL and Rainbow™ colored protein molecular weight markers were purchased from Amersham Life Science, Buckinghamshire, England. The β-agonist, L-644,969 (R,R isomer of 6-amino-alpha{[(1-methyl-3-phenylpropyl) amino] methyl}-3-pyridine methanol dichloride), was a gift from Merck Research Laboratories (Rahway, NJ. U.S.A.).

5.2.2. Animals and Management

A total of 16 Suffolk-cross 5-mo old growing wether lambs weighing 30.2 ± 2.06 kg were randomly allocated to one of four treatment groups (WN, WA, CN and CA) in a 2 x 2 factorial design. The factors were temperature (warm {W} 20 °C; or cold {C} 0 °C) with (A, 4 mg kg^{-1} diet) or without (N) beta-agonist L-644,969 treatment. Animals were housed in individual floor pens in temperature controlled rooms with continuous lighting protocols. Animals were fed an alfalfa based diet (18.32 MJ GE kg^{-1} DM; 20% CP) along

with a barley-based concentrate diet (17.35 MJ GE kg^{-1} DM; 16% CP) that also served as carrier for the beta-agonist. The concentrate diet contained 77.4 % Barley grain, 10% corn grain, 10 % soybean meal, 1.2 % calcium phosphate, 1.2% trace mineralized salt and 0.2% vitamin ADE. All animals were adapted to the experimental protocols for a one-week period in two separate rooms at 20 °C at which time they received alfalfa pellets on *ad libitum* basis and concentrate diet (without agonist) at 26.7 g DM/d $kg^{0.75}$. The experimental protocols were approved by the Faculty Animal Policy and Welfare Committee and animals were cared for according to guidelines of the Canadian Council on Animal Care (1993).

On the last day of the adaptation period all animals were shorn and within 3 d the temperature of one animal room was gradually lowered to 0 ± 1 °C and the other room was maintained at 20 ± 1 °C. All animals were weighed weekly. In the cold environment feed supply was the same as in the warm environment so as to remove any confounding effect of differences in intakes between the two thermal environments. Water and cobalt iodized salt blocks were continuously available to all the animals.

During the third to fourth week of the experiment, animals were transferred into individual metabolism crates and oxygen consumption by the animals was measured by indirect calorimetry. A nitrogen balance study was also conducted by total fecal and urinary collection. Blood samples were collected from the animals weekly for plasma hormone analyses. When the animals were 5 wk on the experiment (excluding the adaptation period) they were killed by captive bolt stunning and rapid exsanguination. Skeletal muscle (*longissimus dorsi* and *biceps femoris*) and adipose tissue (subcutaneous, mesenteric and perirenal) samples were rapidly collected (within 10 min of stunning),

frozen in liquid nitrogen and stored at – 80 °C for analyses. A portion of each fresh adipose tissue sample was rapidly processed and used for an *in vitro* fatty acid synthesis experiment as described below.

5.2.3. *In Vitro* Fatty Acid Synthesis

Adipose tissues were excised from animals within 10 min after stunning and thin tissue slices were prepared free hand with a scissors. Each tissue slice (~150 mg) was incubated in a flask in the presence of 1 μCi [1-^{14}C]acetate, 10 mM sodium acetate and 5 mM glucose in 3 ml Krebs-Ringer-bicarbonate buffer (pH 7.4) containing 119 mM NaCl, 4.82 mM KCl, 1.25 mM $MgSO_4$, 1.24 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 2.0 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) under 95% O_2 and 5% CO_2 in a metabolic shaker. Incubation media also contained 40 mg BSA and 0.1 unit insulin / ml of buffer. Also, blank preparations were made that included the radioisotope and 0.1 ml of 70% perchloric acid prior to adding the tissue to allow for correction for radioactivity artifact. All *in vitro* incubations lasted 2 h and reactions were terminated (except blanks) by addition of 0.1 ml of 70% perchloric acid.

Following termination of incubation, tissue samples were removed from incubation media, quickly rinsed in fresh non-radioactive buffer, placed in microfuge tubes and snap frozen in liquid nitrogen. All samples were kept at – 80 °C until analyzed. Aliquots (100 μl) of the incubation media were counted in liquid scintillation cocktail (EcoLite) for determination of specific radioactivity of [^{14}C]acetate.

5.2.4. Lipid Extraction

Total lipids were extracted according to Folch et al. (1957) as described in Chapter three. Briefly, an individual sample was placed into a 50 ml tube with a teflon lined screw cap and homogenized in 20 ml chloroform:methanol (2:1 v/v). Homogenate samples were kept overnight at 37 °C in a metabolic shaker to allow for complete extraction of tissue lipids and then transferred into graduated cylinders (with stoppers), equilibrated with 5 ml of 0.88 % NaCl solution and left for 8 h in a fume hood. After separation of the mixture, the volume of the bottom layer was recorded and the upper methanol phase was aspirated under gentle water vacuum. Fifteen ml of the bottom chloroform layer was evaporated to dryness in a pre-weighed 20 ml glass scintillation vial under gentle stream of N_2. Dried samples were weighed and resuspended in 10 ml liquid scintillation cocktail (containing 16 g of 2, 5- Diphenyloxazole and 0.4 g of 1, 4-bis[2-(5-phenyloxazolyl)] benzene in 4 liters of Toluene), and radioactivity was counted with a Packard scintillation analyzer (model 1600CA TRI-CARB®; Packard Instruments Co., Downers Grove, IL). Incorporation of [^{14}C]acetate into total lipids was calculated as nmol acetate incorporated per 2 h per 100 mg wet tissue sample from the specific radioactivity of [^{14}C]acetate.

5.2.5. Sample Preparation for Enzyme Extraction

Frozen tissue samples were powdered in a mortar and pestle using liquid nitrogen, and 2 g was homogenized (teflon Potter-Elvehjem homogenizer) for 20 s in three times volume of buffer. The buffer contained, 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5 at 4 °C), 50 mM sodium fluoride, 0.25 M mannitol, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-

tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 5 mM sodium pyrophosphate, 1 mM phenylmethanesulphonyl fluoride, 4 µg/ml each of aprotinin, leupeptin, pepstatin A and soybean trypsin inhibitor, and 1 mM benzamidine. Homogenates were centrifuged using a JA-20 rotor (Beckman) at 14,000 x g for 20 min at 4 °C and the supernatant used for extraction of ACC and FAS.

5.2.6. Acetyl-CoA carboxylase Extraction and Assay

Acetyl-CoA carboxylase extraction and assay were as described in chapter four. Polyethylene glycol 8000 (PEG) was added to 2 ml aliquots of the supernatant as prepared from above step to a final concentration of 2 % PEG. The samples were agitated for 10 min at 4 °C and then centrifuged at 10,000 x g for 10 min in a JA-21 rotor (Beckman Instruments Inc., Irvine, CA). Acetyl-CoA carboxylase protein was precipitated from the supernatant in a 10 % PEG solution, stirred on ice for 10 min and centrifuged as before. The precipitate was then collected and washed with 10 % PEG 8000 in homogenizing buffer. After centrifugation (10,000 x g, 10 min) the pellet was resuspended in another buffer containing 100 mM Tris-HCl (pH 7.5 at 4 °C), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM Na pyrophosphate, 10 % (w/v) glycerol, 0.02 % sodium azide, 4 µg/ml soybean trypsin inhibitor 4 µg/ml each of aprotinin, leupeptin and pepstatin A, and 1 mM benzamidine. The protein content of the resuspended enzyme was measured using bicinchoninic acid reagents.

Activity of ACC was assayed by the $H^{14}CO_3^-$ fixation method (Thampy and Wakil 1985; Lopaschuk et al. 1994). Final concentrations of assay components were 60.6 mM Tris-acetate, 2.12 mM ATP, 1.32 µM β-mercaptoethanol, 50 mM Mg acetate, 10

mM potassium citrate, 1.06 mM acetyl-CoA, 18.18 mM $NaHCO_3$, 0.33 μCi/μmol $NaH^{14}CO_3$ and 1 mg/ml fatty acid free-BSA, pH 7.5. Enzyme preparations were first preincubated for 5 min in a buffer (3:1 v/v) containing 240 mM Tris-acetate, 3 mg/ml BSA (essentially fatty acid free), 20 mM Mg acetate, 40 mM citrate and 5.2 μM β-mercaptoethanol. Acetyl-CoA carboxylase assay reactions were started by addition of 10 μl of preincubated enzyme preparation in a final assay mixture of 165 μl. After 4 min incubation at 37 °C the reaction was stopped by addition of 25 μl of 10 % perchloric acid. Reaction tubes were placed in a dessicator under vacuum to remove unreacted label and tubes were then centrifuged (model J-6M/E and JS-5.2 type rotor; Beckman Instruments Inc., Irvine, CA) at 3500 rpm (2900 x g) for 20 min. After centrifugation 160 μl of the supernatant was transferred into glass scintillation minivials and evaporated to dryness at 80 °C in a fume hood under gentle vacuum. The residue was dissolved in 100 μl of H_20 and mixed with 4 ml scintillation fluid (EcoLite) for determination of radioactivity. Acetyl-CoA carboxylase activity was expressed as nmol $H^{14}CO_3$ incorporated into malonyl-CoA.min^{-1}.mg cytosolic $protein^{-1}$.

5.2.7. Fatty Acid Synthase Assay

Two-ml aliquots of the 14,000 x g supernatant from the sample preparation step were centrifuged in a Solvall® Ultra *pro*80™ (Du Pont Co. DE, USA) ultracentrifuge at 105,000 x g for 60 min to obtain skeletal muscle and adipose tissue cytosol. The supernatant was brought to saturation with saturated ammonium sulfate solution (containing 3 mM EDTA and 1 mM β-mercaptoethanol) and stirred for 60 min on ice. The precipitate was collected by centrifugation at 105,000 x g for 60 min. The pellet was

dissolved in 5 to 10 % of the original volume of the homogenate buffer and was centrifuged briefly in a microfuge tube to remove insoluble protein. The protein content of the supernatant was determined using the bicinchoninic acid protein reagents (Pierce, Rockford, IL, U.S.A.). The supernatant was then used for determination of FAS activity.

FAS activity was assayed according to the method of Nepokroeff et al. (1975) and this measured the malonyl-CoA and acetyl-CoA dependent oxidation of NADPH. The reaction was carried out at 30 °C using a Varian Cary UV-visible automated spectrophotometer (Varian Australia Pty Ltd. Mulgrave, Victoria, Australia). The decrease in absorbance at 340 nm was recorded using a suitable software program and the reaction was linear in the ranges employed. Final concentrations of assay mixture were (per ml) 500 µmol potassium phosphate buffer, 1µmol EDTA, 1 µmol β-mercaptoethanol, 35 nmol acetyl-CoA, 100 nmol malonyl-CoA and 100 nmol NADPH. Fatty acid synthase activity was expressed as nmol palmitate synthesized.min^{-1}.mg enzyme $protein^{-1}$.

5.2.8. Western-Blot Analyses of ACC and FAS

Muscle (60 µg) or 20 to 25 µg of adipose tissue protein extracts were prepared in a sample buffer containing 10 % glycerol, 62.5 mM Tris, pH 6.8, 2% β-mercaptoethanol and 0.025 % bromophenol blue. After being heated in boiling water for 5 min, the samples were subjected to SDS-PAGE using the method of Laemmli (1970). For each tissue set, gels were run in duplicate on the same Bio-Rad Mini-PROTEAN II gel unit (Bio-Rad Laboratories, Missisauga, ON), in the presence of 0.1 % SDS, 25 mM Tris, 192 mM glycine, pH 8.3, at 100 volts for 2.5 h. Membranes were blocked with 10 % skim

milk, and for ACC, one set of membranes was probed with peroxidase labelled streptavidin (Jackson ImmunoResearch Laboratories, Inc.WestGrove, PA). For FAS the second set of membranes were probed with mouse anti-FAS monoclonal antibody (Chemicon International Inc. Temecula, CA) and a secondary goat anti mouse-horse radish peroxidase. An enhanced chemiluminescent detection was performed on the membrane using an ECL™ western blotting kit (Amersham Life Science, Buckinghamshire, England), and quickly followed by autoradiography. Developed images were analyzed using an imaging densitometer (Model GS-670) and a molecular analyst software (Bio-Rad laboratories, Hercules, CA).

5.2.9. Statistical Analyses

Data were analyzed by analysis of variance using the general linear model procedure of SAS (1996). The model included environment, treatment, and environment x treatment effects. All means are expressed as least-square means, and differences between specific treatments were tested using LSD procedure of SAS. Results were considered significant at $p < 0.05$.

5.3. RESULTS

5.3.1. *In Vitro* Fatty Acid Synthesis

Feeding lambs the beta-agonist, L644,969, significantly increased the rate of fatty acid synthesis by 38 % in subcutaneous adipose tissue from cold exposed animals (Figure 5.2-A). In contrast, the compound produced a 27 % decrease ($p < 0.05$) in the rate of fatty acid synthesis of the same adipose tissue when the animals were maintained in the warm

environment. There was no significant difference in the rate of fatty acid synthesis between the control groups in the warm and cold environments in this tissue. In mesenteric adipose tissue (Figure 5.2-B), the rate of fatty acid synthesis in the cold exposed lambs followed the same pattern as seen for subcutaneous adipose tissue. Beta-agonist feeding increased ($p < 0.05$) the rate of fatty acid synthesis in the cold but there was no effect in the warm environment. In perirenal adipose tissue (Figure 5.2-C), the rate of fatty acid synthesis was significantly reduced ($p < 0.05$) by 28 % when L-644,969 β-adrenergic agent was fed to the lambs in the warm environment. In the cold environment, the effect of the β-agonist was not significant.

5.3.2. Enzyme Activity

Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) activities were measured on frozen skeletal muscle and adipose tissue samples collected 10 min after exsanguination. In *longissimus dorsi* muscle, ACC activity (Table 5.1) was depressed ($p < 0.05$) when the β-AR agent was fed to animals in the warm environment but the agonist had no effect in the cold environment. In *biceps femoris* muscle, ACC activity was not altered in response to either cold environmental exposure or β-agonist feeding.

In subcutaneous adipose tissue, feeding the β-AR agonist to sheep in the warm environment reduced ($p < 0.05$) ACC activity by 25 % but the 28 % reduction in the cold environment was not significant. In mesenteric fat depot, β-agonist significantly reduced ($p < 0.05$) ACC activity by more than 40 % in the warm environment but had no effect in the cold exposed animals. In the non-agonist treated groups in the two environments, ACC activity in the cold-control animals was about 27 % lower ($p > 0.05$) compared to

the warm-control animals, with mean activity values of 12.07 ± 1.42 for cold control and 16.72 ± 3.90 nmol min^{-1} mg $protein^{-1}$ for the warm control animals. In perirenal adipose tissue, ACC activity measured in tissue from cold-exposed lambs was higher than that in tissue from the animals in the warm environment ($p < 0.05$). Acetyl-CoA carboxylase activity in the warm-agonist group was lower ($p < 0.05$) than activity in the warm-control group in agreement with the rates of fatty acid synthesis data. However, feeding the beta-agonist, L644,969, to animals in the cold environment increased the activity of ACC in perirenal adipose tissue by 33 %.

The activities of FAS in skeletal muscle and adipose tissue from sheep are presented in Table 5.2. Both environmental temperature exposure and β-AR agonist treatment had some effects on FAS activity in the *longissimus dorsi* muscle. Fatty acid synthase activity was lower in LD muscle from cold exposed sheep but the effect only approached significance ($p = 0.08$). The numerically lower values of FAS activity in the agonist treated groups in the warm and cold environments compared to the control groups in each environment were not statistically significant. There was also no significant effect of environment and the β-AR agent on FAS activity in *biceps femoris* muscle. In contrast to the effects of cold exposure and β-agonist treatment of animals on ACC activity, both cold exposure and β-agonist treatment had no effect on fatty acid synthase activity in subcutaneous and mesenteric adipose tissues. The activity was numerically the same for all treatment groups in subcutaneous adipose tissue. In mesenteric adipose tissue there was a slight decrease in FAS activity in the cold exposed animals following beta-agonist treatment. In perirenal adipose tissue, FAS activity was lower ($p < 0.05$) in the

cold exposed lambs compared to the warm groups. Feeding agonist in the warm environment significantly increased ($p < 0.05$) FAS activity.

5.3.3. Enzyme Protein Abundance

The results of western-blot analyses of ACC protein expression are presented in Figures 5.3 and 5.4 with the densitometric units presented in Table 5.3. In *longissimus dorsi* muscle, two isoforms of ACC protein corresponding to 280,000 and 265,000 Da were recognized. Analyses of the band data showed that the 280 kDa isoform of ACC was highly expressed in the cold exposed animals ($p < 0.05$) compared to the warm control. In the warm environment, feeding β-agonist increased ($p < 0.05$) ACC protein abundance when compared to animals in the non-agonist fed control. There was no significant difference between the cold-agonist and the cold-control groups. However, ACC protein abundance increased in the cold control relative to the warm control. Feeding β-AR agonist in the cold environment significantly decreased the 265-kDa expression but there was no significant difference between the warm control and the warm agonist fed group.

In the *biceps femoris* muscle, the peroxidase-conjugated-streptavidin recognized only one distinct isoform of ACC (280 kDa). There was no significant effect of environmental temperature on the expression of this isoform. However, ACC protein abundance was increased in the agonist group in warm environment by up to 33 % compared to the warm-control group.

In subcutaneous adipose tissue, there was no significant effect of environmental temperature or agonist on immunodetectable ACC protein. However, in the warm environment β-agonist caused a 50 % increase ($p > 0.05$) in ACC protein abundance

compared to the control. In mesenteric adipose tissue, a temperature x agonist interaction approached significance ($p = 0.06$) in which β-agonist treatment in the cold environment reduced ACC protein abundance by 37 % in this tissue. There was no separate effect of temperature or agonist treatment on ACC protein expression in mesenteric fat depot. In perirenal adipose tissue, β-AR agonist significantly reduced ACC protein expression in the cold but had no effect in the warm environment.

Results of FAS protein expression in adipose tissue were fairly similar to those of ACC in the same tissues. As shown in Figure 5.5 and Table 5.4 the pattern of FAS protein expression in the two skeletal muscles was not uniform. In *longissimus dorsi*, β-agonist significantly increased ($p < 0.05$) FAS protein abundance in the cold exposed animals but there was no significant effect in the warm environment. For the *biceps femoris* muscle feeding β-agonist increased ($p > 0.05$) FAS protein expression in the warm environment.

In subcutaneous adipose tissue there was no significant effect of the environment and agonist treatment on the expression of the FAS protein. However, numerically lower values were seen in the two environments following β-agonist treatment. In mesenteric adipose tissue, whereas the beta-agonist reduced ($p < 0.05$) FAS protein during cold exposure, there was no significant difference between the control and the β-agonist treated group in the warm environment. In perirenal fat, feeding β-agonist to the animals reduced ($p < 0.05$) FAS protein expression in the two environments but there was no significant effect of environment.

5.4. DISCUSSION

Cold temperature exposure causes increased release of endogenous catecholamines (Sasaki and Weekes 1984) leading to increased tissue responses. Hormonal changes that may follow cold temperature exposure may also have effects on fatty acid synthesis in white adipose tissue. Thus, changes in insulin for example will stimulate lipogenesis in adipose tissues while catecholamines will inhibit lipogenesis. The mechanisms of the inhibitory effects of catecholamines are thought to involve β-AR-mediated phosphorylation and inactivation of ACC in adipose tissue (Lee and Kim 1978; Brownsey et al. 1979). In the liver, there appears to be an alternate mechanism of inactivation of ACC by catecholamines and its agonists through the α-adrenoceptor mechanism (Ly and Kim 1981).

The present studies showed that β-AR agents regulated fatty acid synthesis in adipose tissues and the effects on these tissues were manifested in opposite directions in tissues from animals adapted to cold environments. L-644,969 is structurally related to ractopamine. Ractopamine has been found to stimulate lipolysis and depress lipogenesis by rat adipose tissue *in vitro* (Hausman et al. 1989). The decreased rates of fatty acid synthesis by L-644,969 in the warm environment are in agreement with this and other studies (Müller-Wieland et al. 1994; Zhang and Grieve 1995). However, one investigation (Liu et al. 1994) indicated that ractopamine, when fed to pigs, had little or no effect on carcass fat deposition or on adipocyte lipogenic rates measured *in vitro*. Another study showed that ractopamine caused a decrease in carcass fat but little or no decrease in the daily rate of fat deposition (Dunshea et al. 1993a,b). It is possible that the different effects of L-644,969 on fatty acid synthesis observed in adipose tissues in the

current study may be entangled with some or even most of the ultimate effects resulting from secondary events caused by hormonal or physiological responses of tissues to the β-AR agonist administered (Mersmann 1998). For example, glucose and insulin were included in the *in vitro* incubation media. Insulin usually will potently increase lipogenesis in tissues. The concentration of insulin used in the incubation media may be different from the physiological concentration of the hormone. It is likely that this could have altered the properties of the adrenergic-mediated effects, and therefore could have implications on the mechanism of action of the beta-adrenergic agonist on rate of fatty acid synthesis *in vitro*.

The mechanisms for the different effects of β-AR agonist on rates of *in vitro* fatty acid synthesis in adipose tissues of animals acclimated to different environmental temperatures are uncertain but may proceed through the G_s proteins to the activation of adenylyl cyclase to produce cAMP. Because of the similarity in the structure of β-AR and α-adrenoceptor it is also possible that there could be some activation of α-adrenoceptor (Mersmann 1998) by the β-AR agonist L-644,969 during cold exposure. α-adrenergic stimulation in adipose tissue inhibits lipolysis (Kather et al. 1980) which may lead to a stimulation of lipogenesis.

The present study demonstrated that fatty acid synthesis in adipose tissue from lambs was dependent on the environmental temperature to which the animals were adapted. In the agonist fed groups, it was also shown that the rates of fatty acid synthesis was inversely related to the acclimation temperature of the animals. These results are in agreement with those of Buckley and Rath (1987) who showed that ^3H incorporation into white adipose tissue of rats was increased during long-term cold exposure. However, the

increased rates of synthesis were less than the 5 to 6 times obtained by Trayhurn (1981) in adipose tissue from mice acclimated to a cold temperature compared to warm control.

During prolonged cold exposure of animals, β-AR agonist administration can increase blood flow to adipose tissues, and thereby enhancing the rates of delivery of lipogenic substrates to these tissues. The increased substrate delivery could have contributed to the enhanced rates of fatty acid synthesis observed in the various adipose tissues of the cold exposed sheep in the current study.

The rate of *in vitro* fatty acid synthesis in skeletal muscle was not determined in this study but should probably reflect changes in the activity of the enzymes that regulate this pathway. The current study demonstrated no significant effect of environmental temperature on ACC activity in *longissimus dorsi* muscle in agreement with the previous study reported in Chapter four. However, if β-AR exert their partitioning effect on muscle by reducing fat synthesis in the warm environment, then the change in ACC activity in *longissimus dorsi* muscle of L-644,969 treated / warm-acclimated animals is consistent with a reduction of fat, and, an increased *longissimus dorsi* area in sheep (Galbraith et al. 1997).

Both *longissimus dorsi* and *biceps femoris* muscles were selected for this experiment because they are large and accessible for obtaining samples rapidly from approximately the same area in different animals. In both muscles the αW-fibre type accounted for just over 50 % of the total fibres with the remaining percentage divided approximately equally between the αR- and βR-fibre types (Johnston et al. 1981). The fact that no significant effects of β-AR agonist or environmental temperature were obtained for the *biceps femoris* muscle compared to reduced ACC activity in *longissimus*

dorsi muscle from warm acclimated sheep may relate to the fact that only one isoform (the non-inducible constitutive form) of ACC was identified in the *biceps femoris*.

The current data also showed that changes in the rates of fatty acid synthesis measured in fresh adipose tissues were paralleled by similar changes in ACC activity measured in frozen adipose tissues. These data are consistent with those of McCormack and Denton (1977) who showed that high activities of pyruvate dehydrogenase and ACC were consistent with high rates of fatty acid synthesis in brown adipose tissue of cold-acclimated rats. However, in mesenteric adipose tissue of warm acclimated sheep, although the use of the β-AR agonist decreased ACC activity there was no parallel change in the observed rate of fatty acid synthesis.

In this study, a pattern of increased ACC activity in adipose tissue from animals adapted to chronic cold exposure was reported. During cold exposure when energy needs of the tissues are higher it is possible for ACC activity to decline in the short-term so as to relieve inhibition on the β-oxidation pathway. With continuous acclimation to the cold environment ACC activity will probably increase so as to furnish more substrates required for heat generation.

The general effects of β-AR agonists in animals are increased muscle protein accretion, improved feed efficiency and reduced carcass fat. The mechanisms responsible for the decreased carcass fat are still much in question but may involve the regulation of lipogenic enzymes. Decreased ACC activity has been reported in adipose tissues of rats treated with β-AR agonist BRL 26830 (Wilson 1989). Results in the current study showed that the β-AR agonist, L-644,969, reduced ACC activity in adipose tissue of warm-acclimated animals but had no significant effect on FAS under the same conditions

except in perirenal adipose tissue. There is much evidence that the mechanism involved in the reduction of ACC activity in adipose tissue involves AMP-dependent protein kinase, thus increased dephosphorylation of ACC would result in the activation of this enzyme (Allred and Reilly 1997). Substantial evidence now indicates that the phosphorylation of the serine-79 of ACC dramatically decreases the V_{max} of the enzyme and that this is catalyzed by the AMP-activated protein kinase (Davies et al. 1990; Haystead et al. 1990; Ha et al. 1994; Witters et al. 1994; Kudo et al. 1995). It is apparent that ACC is not controlled by a single mechanism but rather the interaction of several regulatory strategies.

Enzyme activity is determined by long term systems which control the quantity of the active and activatable enzyme, as well as short-term regulatory systems which affect enzyme catalytic efficiency (Hillgartner et al. 1995; Allred and Reilly 1997). In isolated adipocytes treated with epinephrine, Brownsey et al. (1979), showed that ACC was phosphorylated and inactivated which seems to indicate that ACC was regulated by cAMP. It was also observed that injection of epinephrine into rats resulted in phosphorylation and inactivation of ACC isolated from epididymal adipose tissue (Lee and Kim 1979). Because both phosphorylation and inactivation of the enzyme were blocked by propranolol, a β-AR blocking agent, the data in the present study may suggest that the regulation of the activity of ACC by the β-AR agonist L-644,969, occurred via a cyclic-AMP-linked protein kinase cascade.

Fatty acid synthase activity is regulated by both diet and hormones (Katsurada et al. 1990; Fukuda et al. 1992; Clarke and Jump 1993). In Chapters two and four, it was shown that FAS activity in sheep was affected by acclimation temperature in

subcutaneous adipose tissue. The current data however found that the combination of β-agonist and environmental temperature produced no additive change in FAS activity in *longissimus dorsi* and *biceps femoris* muscles. The absence of any significant effect of environmental temperature or of L-644,969 agonist on FAS activity in adipose tissues of sheep (except in perirenal adipose tissue where the effect of environment was significant) even when ACC activity was altered, was not consistent with the data on rates of fatty acid synthesis in these same tissues. The reasons for the differences in response of ACC and FAS to the treatments are not apparent other than possible differences in responses to secondary effects of environmental temperature and β-AR agonist on the enzymes. The lack of co-ordinated regulation between these two enzymes may suggest that common regulatory mechanisms are not involved in adipose tissues of lambs.

In previous reports the concentration of the 265-kDa isoform of ACC has been measured in various tissues with various immunological methods (Bianchi et al. 1990; Iverson et al. 1990; Thampy and Koshy 1991; Spencer et al. 1993). By western-blot analyses a 280-kDa and a 265-kDa isoform of ACC were detected in *longissimus dorsi* muscle using peroxidase- conjugated-streptavidin. However, in *biceps femoris* muscle, only the 280-kDa isoform of ACC was clearly detected. It is not known why the two types of skeletal muscle showed differences in the expression of the ACC proteins. On the other hand, it was possible that the ACC-265 isoform was also expressed in *biceps femoris* in low concentrations that were below the detection limit of the analytical methods employed in the current study. In general, the expression of ACC protein in these tissues was reflected in the activity of the enzyme. The enzyme protein content as determined by western-blot, and the activity of ACC in adipose tissue followed opposite

patterns, except in perirenal adipose tissue from the warm acclimated sheep where β-agonist significantly reduced both the activity and the amount of enzyme protein when compared to the control group. The lack of similarity between the activity and the western-blot data suggests that the changes observed in ACC activity were not likely due to changes in the amount of enzyme protein.

Unlike the FAS activity that showed little or no response to the treatments, western blot analyses of the enzyme showed some treatment effects in all tissues examined. The effects of environmental temperature and β-AR agonist treatment on *longissimus dorsi* FAS protein are consistent with that of FAS activity in which decreased protein expression was accompanied by similar decrease in enzyme activity. In the *biceps femoris* muscle, the pattern of FAS protein expression was somewhat different from that of FAS activity, however there were no significant treatment effects on either the activity or the protein expression. In general, the subtle change in FAS protein expression in adipose tissue was probably not enough to induce any significant change in enzyme activity in the same tissues.

In conclusion, data in the present study suggest that multiple isoforms of ACC are present in *longissimus dorsi* muscle but only one isoform was expressed in the *biceps femoris*. The 280-kDa isoform in *longissimus dorsi* muscle was responsive to environmental temperature and not the 265-kDa isoform. This data therefore confirms results in previous section (chapter four) that showed an elevation of the 280-kDa isoform in the cold environment. The response of this isoform to environmental temperature may implicate it in the regulation of substrate oxidation in the skeletal tissues (Saddik et al. 1993; Lopaschuk et al. 1994).

By feeding a β-AR L-644,969, the decrease in rates of in vitro fatty acid synthesis in subcutaneous and perirenal adipose tissue in the cold environment paralleled a similar decrease in ACC activity but with little or no effect on FAS activity. Based on the in vitro fatty acid synthesis data, it seems likely that the influence of β-agonist on adipose tissue lipogenesis was temperature specific, producing a decrease in the rates of fatty acid synthesis in tissue from the warm acclimated animals but an increase in tissue from the cold acclimated animals. It is likely that the endogenous catecholamines and the increased energy requirement of the animals maintained in the cold environment could be modulating the adrenoceptor processes that are initiated by the exogenous adrenergic agonist.

Table 5.1. Effect of environmental temperature and beta-adrenergic agonist (0 or 4 mg kg^{-1}) on acetyl-CoA carboxylase activity in skeletal muscle and subcutaneous, mesenteric and perirenal adipose tissues from sheep.

	Warm-Control	Warm-Agonist	Cold-Control	Cold – Agonist	SE[z]
L. dorsi	0.603 [a]	0.292 [b]	0.483 [ab]	0.401 [ab]	0.10
B. femoris	0.352	0.433	0.357	0.455	0.13
Subcutaneous	16.92 [a]	12.63 [a]	13.75 [a]	9.94 [b]	2.83
Mesenteric	16.72 [a]	9.52 [b]	12.07 [ab]	13.92 [a]	2.53
Perirenal	10.25 [a]	3.85 [b]	11.13 [a]	14.83 [a]	2.70

ACC activity is expressed as nmol $NaH^{14}CO_3$ incorporated into stable malonyl-CoA.min^{-1}.mg $protein^{-1}$. Number of animals per mean = 4.

[z]Pooled standard error of least square means.

[a, b]Means within rows with different superscripts are significantly different, $p < 0.05$.

Table 5.2. Effect of environmental temperature and beta-adrenergic agonist L-644,969 on fatty acid synthase activity in skeletal muscle and subcutaneous, mesenteric and perirenal adipose tissues from sheep.

	Warm-Control	Warm-Agonist	Cold-Control	Cold – Agonist	SE[z]
L. dorsi	0.830	0.584	0.414	0.378	0.161
B. femoris	0.552	0.459	0.574	1.006	0.151
Subcutaneous	19.92	20.57	20.79	20.93	3.31
Mesenteric	16.96	16.61	16.27	14.70	3.50
Perirenal	30.10 [b]	39.80 [a]	22.72 [c]	20.69 [c]	5.75

FAS activity was determined as described under 'Materials and Methods' and expressed as nmol palmitate synthesized.min^{-1}.mg protein^{-1}. Number of animals per mean = 4.

[z]Pooled standard error of least square means.

[a, b, c] Means within rows with different superscripts are significantly different, $p < 0.05$.

Table 5.3. Effect of environmental temperature and beta-adrenergic agonist on ACC protein expression in skeletal muscle and subcutaneous, mesenteric and perirenal adipose tissues from sheep.

	Warm-Control	Warm-Agonist	Cold-Control	Cold – Agonist	SE[z]
L. dorsi (280-kDa)	6.75 [b]	9.94 [a]	10.20 [a]	10.10 [a]	0.76
L. dorsi (265-kDa)	4.93 [b]	4.53 [a b]	5.46 [a]	3.19 [b]	0.60
B. femoris (280-kDa)	2.71 [b]	4.05 [a]	3.92 [a]	4.99 [a]	0.59
Subcutaneous fat	6.43	9.81	7.10	5.77	2.01
Mesenteric fat	10.50 [a]	10.38 [a]	10.68 [a]	6.53 [b]	1.78
Perirenal fat	4.78 [b]	6.13 [a b]	7.83 [a]	2.64 [b]	1.64

Values are least square means of arbitrary densitometry units. For *L. dorsi*, *B. femoris*, subcutaneous and mesenteric, number of animal (n) per mean = 4 except in warm-control treatment where n = 3. For perirenal warm- and cold-control, n = 4, and warm- and cold-agonist, n = 3.

[z]Pooled standard error of least square means for treatment effect.

[a,b]Means in rows followed by different superscripts are significantly different ($p < 0.05$).

Table 5.4. Effect of environmental temperature and beta-adrenergic agonist on FAS protein expression in skeletal muscle and subcutaneous, mesenteric and perirenal adipose tissues from sheep.

	Warm-Control	Warm-Agonist	Cold-Control	Cold – Agonist	SE[y]
L. dorsi	3.66 [a]	3.36 [a]	1.69 [b]	4.01 [a]	1.31
B. femoris	2.60	5.29	9.26	5.26	3.21
Subcutaneous fat	25.10	21.10	22.93	19.61	2.46
Mesenteric fat	13.14 [a b]	14.57 [a b]	16.92 [a]	9.71 [b]	2.26
Perirenal fat	17.98 [a]	14.67[b]	19.99 [a]	12.98 [b]	1.69

Values are least square means of arbitrary units. For *L. dorsi*, *B. femoris*, subcutaneous and mesenteric, number of animal (n) per mean = 4 except in warm control treatment where n = 3. For perirenal, warm- and cold-control, n = 4, and warm- and cold-agonist, n = 3.

[z]Pooled standard error of least square means for treatment effect.

[a,b] Means in rows followed by different superscripts are significantly different ($p < 0.05$).

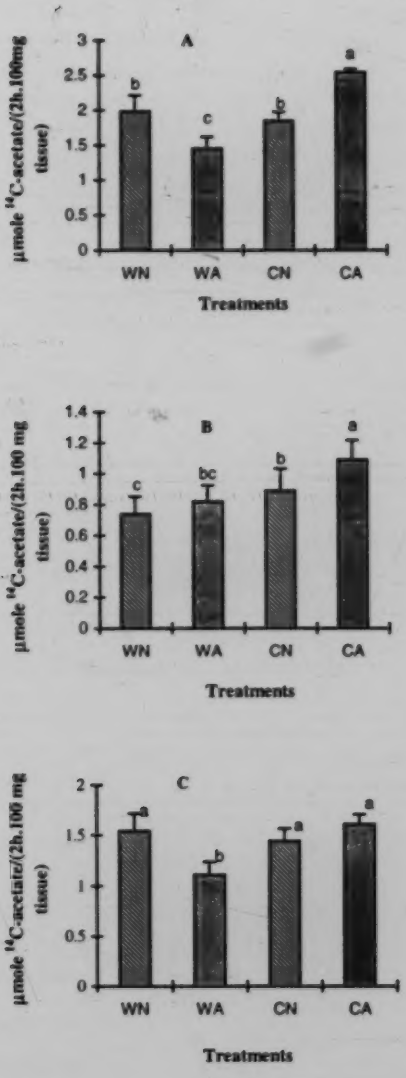

Figure 5.2. The effect of environmental temperature exposure and beta-adrenergic agonist treatment of sheep on rates of fatty acid synthesis in adipose tissues from sheep.

Panel A is subcutaneous, B is mesenteric and C is perirenal adipose tissue. Values represent means ± SEM. [abc] Bars with different notations are significantly different ($p < 0.05$). WA, CA = warm- or cold-agonist; WN, CN = warm- or cold-control treatments, respectively. n = 4 animals per plotted value.

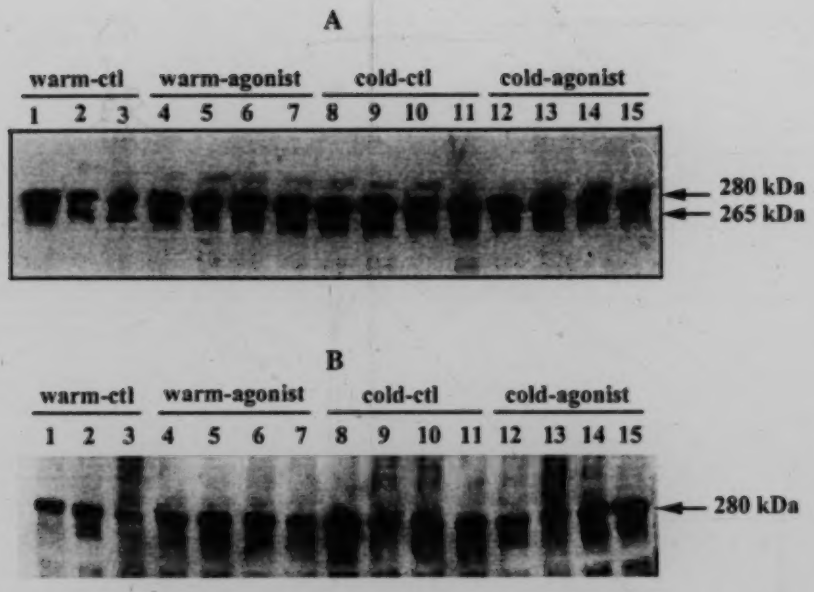

Figure 5.3. Western-blot analyses of ACC protein abundance in *longissimus dorsi* (panel A) and *biceps femoris* (panel B) muscles from sheep exposed to different temperatures with or without beta-agonist.

Western-blot was performed as described under Materials and Methods. SDS-PAGE was carried out by loading 60-µg protein sample into each lane and transferred to a nitrocellulose membrane. After blocking the membranes with 10 % skim milk they were incubated with peroxidase-labeled streptavidin. Texts above the lanes describe treatment combinations. Ctl = control.

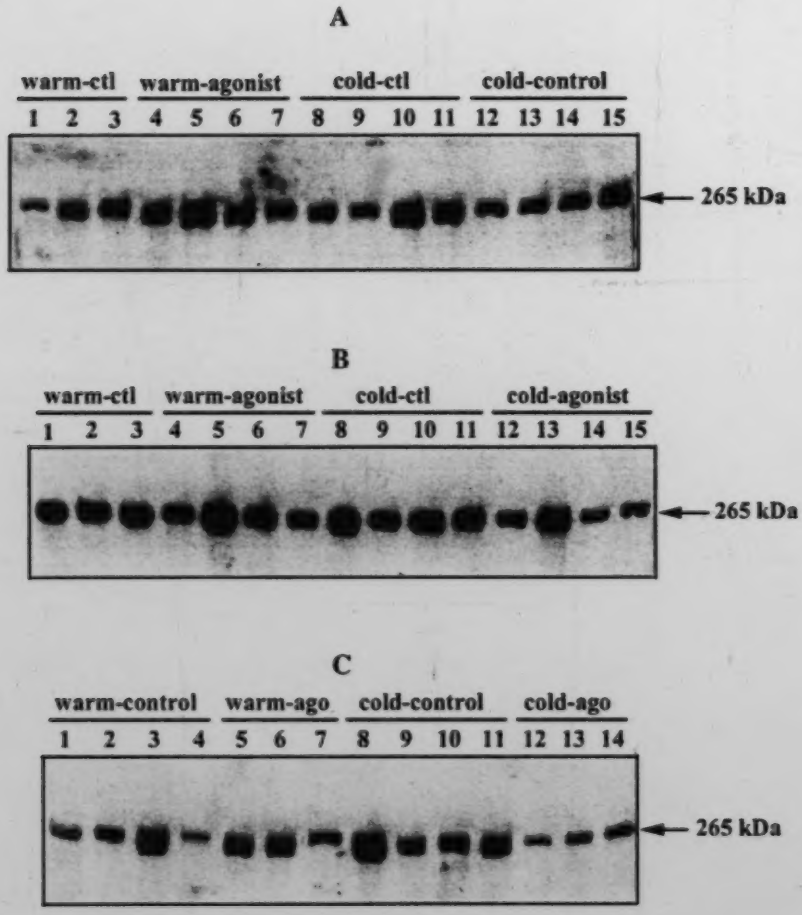

Figure 5.4. The effects of environmental temperature and beta-agonist L-644,969 on ACC protein abundance in adipose tissues from wether lambs.

Panels A, B and C are from subcutaneous, mesenteric and perirenal adipose tissues, respectively. Subcutaneous or perirenal (20 μg), or 25 μg of mesenteric adipose tissue total protein were applied to each lane. ACC was detected using peroxidase-conjugated streptavidin as described under Materials and Methods. Texts above the lanes describe treatment combinations. Ctl = control, and ago = agonist.

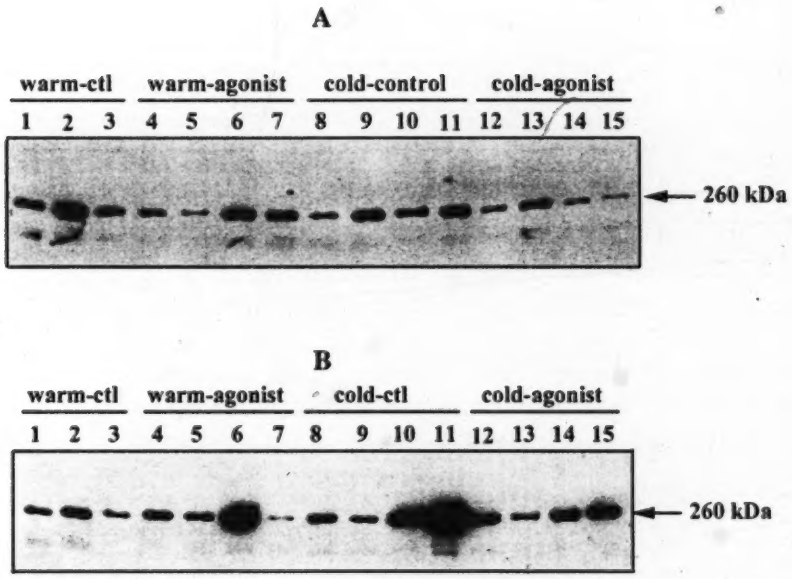

Figure 5.5. The effect of environmental temperature beta-adrenergic agonist on FAS protein abundance in *Longissimus dorsi* (panel A) and *Biceps femoris* (panel B) skeletal muscles from wether lambs.

SDS-PAGE was carried out by loading 60-µg protein sample into each lane. Blots were probed for FAS using mouse anti-FAS monoclonal antibody and a secondary goat anti-mouse horseradish peroxidase. Bands shown correspond to 260,000 Da. Texts above lanes describe treatment combinations. Ctl = control.

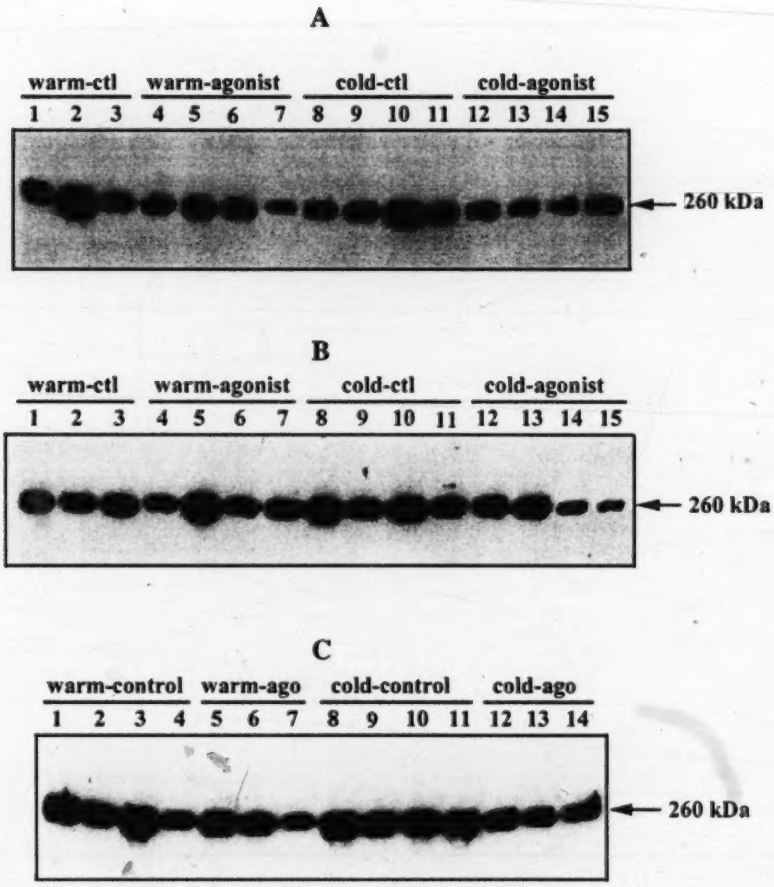

Figure 5.6. The effects of environmental temperature and beta-adrenergic agonist on FAS protein expression in adipose tissue from wether lambs.

Panels A, B and C are from subcutaneous, mesenteric and perirenal adipose tissues respectively. Text above the lanes describes each treatment. 20-μg of subcutaneous or perirenal, or 25-μg of mesenteric fat total protein were applied to each lane and FAS detected as described for Figure 5.5. Bands shown correspond to 260,000 dalton MW. Texts above the lanes describe treatment combinations. Ctl = control, and ago = agonist.

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6. GENERAL DISCUSSION AND CONCLUSIONS

The thermal environment, by modifying energy demands, can elicit changes in metabolic processes within the animal. These may include changes in responsiveness of tissues to dietary factors and hormones, thereby influencing the sensitivity of metabolic regulation. Fatty acid synthesis is one of those metabolic pathways whose rate can be subject to nutritional and hormonal influences. The regulation of fatty acid synthesis in this regard will depend more or less on the regulatory role of the enzymes of the biosynthetic pathway. In this thesis, the influence of thermal environment, diet and a β-adrenergic agonist on the fatty acid biosynthetic pathway, including the key enzymes regulating the pathway was investigated in sheep. This Chapter summarizes the major conclusions which pertain to the major hypotheses that were tested in different parts of the study.

6.1. Expression of Lipogenic Enzymes in Ovine Tissues

Acetyl-CoA carboxylase and FAS play pivotal roles in regulating the rate of fatty acid synthesis in animal tissues. Studies on the regulation of lipogenic enzyme expression have been conducted using a variety of approaches, many of which have investigated nutritional and hormonal regulation of the gene expression and activity of the lipogenic enzymes (e.g., Clarke and Jump 1994; Girard et al. 1994; Iritani et al. 1996; Kim and Freake 1996). In the current studies, the properties of ACC and FAS present in ovine tissues were investigated using a combination of *in vitro* activity determinations and immunological approaches and their relationships to rates of fatty acid synthesis.

By use of western-blotting, the total activity of ACC in *biceps femoris* muscle, liver and adipose tissue of sheep was accounted for by the presence of only one isoform

of the enzyme. Analysis of *longissimus dorsi* (LD) muscle indicated the presence of two isozymes of ACC each with a different molecular mass contributing to the measured activity as presented in Chapters four and five. Evidence in the research literature (Lopaschuk et al. 1994; Trumble et al. 1995) supports the existence of more than one isoform of ACC in heart and skeletal muscles from other species.

Physiological conditions that cause a decrease in ACC activity and malonyl-CoA levels in muscle tissues are accompanied by accelerated rates of fatty acid oxidation in the liver and heart (Awan and Saggerson 1993; Saddik et al. 1993; Lopaschuk et al. 1994; Winz et al. 1994; Wang et al. 1996). This suggests that the presence of an additional isoform in these skeletal tissues is necessary to catalyze the synthesis of malonyl-CoA, a potent inhibitor of the carnitine palmitoyltransferase I system, which in turn, regulates mitochondrial fatty acid oxidation. Indeed the second isoform of ACC (280-kDa) has been purified (Trumble et al. 1995) and its gene cloned (Widmer et al. 1996) and has been implicated as the ACC that controls fatty acid oxidation (Saddik et al. 1993; Winder et al. 1995; Wang et al. 1996).

Data from the current studies indicate that the two isozymes of ACC in LD muscle were differentially expressed. The 265 kDa isoform was lower in abundance compared to the 280 kDa isoform in the same tissue. The 265 kDa isoform abundance in LD muscle was also lower when compared to the expression of ACC protein of similar mass in adipose tissues that are traditionally lipogenic. The activity and protein expression of the two enzymes in the skeletal tissues were generally not altered by dietary treatments or thermal environment in which the animals were maintained in all the experiments reported herein except when the animals were fed a β-AR agonist. The

general pattern of lipogenic enzyme activity in skeletal muscle was less than 5 % of the activity described in the adipose tissues studied. These results are consistent with the *in vitro* rates of fatty acid synthesis reported in Chapter three where the rate of incorporation of [1-^{14}C]acetate into tissue lipids by skeletal muscle was also 5 % of the rates measured in the adipose tissue. The close relationship between the relative enzyme activity measured in different tissues and the corresponding relative rates of fatty acid synthesis in those tissue, in my opinion, suggests that the rates of fatty acid synthesis in any tissue are predetermined or limited by the activity of the synthesizing enzymes in that tissue.

The *in vivo* studies of Ingle et al. (1972) suggested that in non-lactating sheep, adipose tissue was responsible for more than 90 % of the whole body fatty acid biosynthesis. The current study also supports the concept that most lipogenesis occurs in adipose tissue in growing sheep. As indicated by the results of the two *in vitro* studies (Chapters three and five), it appeared that the subcutaneous adipose tissue was more active at incorporating [1-^{14}C]acetate into tissue lipids when compared to the other two adipose tissue depots (i.e. mesenteric and perirenal). This conclusion is in agreement with that of Ingle et al. (1973). A similar conclusion was made by Scott and Prior (1980) that subcutaneous adipose tissue appeared to have more enzymatic (lipogenic) activity than perirenal adipose tissue. de la Hoz and Vernon (1993) also showed that the rate of fatty acid synthesis per adipocyte varied markedly amongst individual non-lactating sheep and that adipocytes from subcutaneous adipose tissue possess the greatest rate of fatty acid synthesis compared to omental or popliteal fat. In the same study, the rates of fatty acid synthesis were significantly greater in all three adipose depots in wether lambs than in corresponding depots in mature ewes. However unlike in non-lactating ewes, the rate of

fatty acid synthesis in lambs was be about 25 % higher in omental compared to subcutaneous adipose tissue.

6.2. Nutritional Regulation of Fatty Acid Synthesis in Sheep

The pathway of fatty acid synthesis is known to be under complex nutritional and hormonal controls (Volpe and Vagelos 1976). Generally, a high carbohydrate fat-free diet stimulates lipogenesis whereas fasting and dietary lipid both have inhibitory effects on rates of fatty acid synthesis. Scott and Prior (1980) have shown that increased dietary energy density significantly elevated subcutaneous adipose tissue FAS activity in small-type (Angus x Hereford reciprocal crossbred) but not in the large-type steers. However, ACC activity in subcutaneous and perirenal adipose tissues did not respond consistently to changes in dietary energy density as observed for FAS.

In the study reported in Chapter two, feed restriction reduced the activity of ACC and FAS in both subcutaneous and mesenteric adipose tissues indicating that the amount of nutrient supply to these tissues probably regulated the enzymes. The results also showed that the protein expression of the two enzymes was depressed in subcutaneous adipose tissue as a result of feed restriction. The pattern was different for protein expression in mesenteric adipose tissue where FAS, but not ACC, was depressed by feed restriction. The lack of effect of dietary restriction on ACC protein expression in mesenteric adipose tissue suggests that ACC was regulated in a different manner in this tissue.

In the experiment just referred to above, differences in feed intake (1.53 kg d^{-1} for restricted-fed vs 3.38 kg d^{-1} for *ad libitum* fed sheep) produced a reduced growth rate in

the restricted group (0.11 kg d^{-1} vs 0.24 kg d^{-1} in *ad libitum*) even though there was no difference in feed efficiency (0.07 kg intake/kg gain in both restricted and *ad libitum*). The reduced growth rate as indicated by the average daily gain of the restricted fed animals (Ekpe 1998) was expected to reduce the rates of fatty acid synthesis. Thus, a decrease in activity of the lipogenic enzymes in adipose tissue of the restricted-fed lambs is consistent with their low level of anabolism. In a similar study with sheep that were fed but losing weight (Prior 1978), reduced rates of fatty acid synthesis were observed from both lactate and acetate. When dietary energy supply is inadequate to meet the maintenance requirements of the restricted fed animals a negative energy balance ensues which may result in a high rate of mobilization of adipose tissue lipids to meet the energy deficit. In addition to the dietary energy deficit, adipose tissue may interact with the hormonal status such as reduced insulin and increased levels of T_3 and glucagon to modulate the lipogenic capacity in adipose tissue during times of low energy balances in sheep.

Furthermore, in circumstances where the intake of feed is restricted, as in this study, the release of norepinephrine (NE) from the sympathetic nervous system (SNS) may be increased, theoretically to conserve energy (Landsberg 1990). In rats a reduction of NE synthesis in or release from the SNS caused an increase in the accretion of body fat (Dulloo and Miller 1985). This suggests that the SNS may adapt by increasing NE synthesis or output to regulate the rates of fatty acid synthesis when feed supply is restricted and correspondingly have an effect on the lipogenic enzymes. This adaptation of SNS however, may be secondary to the regulatory actions of insulin and (or) glucagon or thyroid hormones on lipogenesis. During lactation, when body energy balance is a

major factor in supplying energy to support milk synthesis, decreased lipid synthesis plus enhanced lipolysis have been shown to lead to a substantial loss of adipose tissue lipid reserves (Vernon 1989; McNamara 1991), and insulin is one of the endocrine factors implicated in both lactating and non-lactating sheep to regulate adipose tissue lipogenesis and lipolysis (McNamara 1991; de La Hoz and Vernon 1993).

Apart from feed restriction, alteration in fat content of ruminant diets is another example of the complex effects of dietary nutrients on adipose tissue lipogenic capacity, especially when the fat source is protected from rumen biohydrogenation (McNamara et al. 1995). Chilliard et al. (1991), and Gagliostro and Chilliard (1991) showed that increases in dietary intake of fat or infusion of fat directly into the abomasum or duodenum of dairy cattle decreased adipose tissue lipogenesis and esterification but had little effect on rates of lipolysis.

In sheep, studies have shown that increasing the lipid content of the diet from 4 to 8 % with protected tallow resulted in decreases in rates of fatty acid synthesis, glucose and acetate oxidation and in the activities of several lipogenic enzymes (Vernon 1976). In another study with sheep, Hood et al. (1980) showed that the addition of safflower oil with formaldehyde-treated casein to the diet of 5-yr old wethers resulted in a depression of lipogenesis *in vitro* and *in vivo*. They also showed that in both *in vitro* and *in vivo* studies, perirenal adipose tissue from sheep receiving a lipid supplement was less active in lipogenesis than subcutaneous adipose tissue. All of these studies are in agreement with the results of the current studies presented in Chapters three and four on supplementation of the sheep diet with a lipid source, with a consequent reduction in the rates of fatty acid synthesis in all three adipose tissue depots *in vivo*. The reduction in the

rates of lipogenesis in response to dietary fat can probably be attributed to increased availability of free fatty acids in adipose tissues since fatty acids (especially polyunsaturated fatty acids) are known to inhibit fatty acid synthesis in adipocytes.

The relative change in the rates of fatty acid synthesis *in vivo* as a result of dietary fat supplementation seemed to be more closely related to changes in FAS activity in the tissues than to changes in ACC activity. As presented in Chapter four, the activity of FAS in all adipose tissues was significantly reduced ($p < 0.05$) by dietary fat in agreement with the *in vivo* studies that showed depressed rates of fatty acid synthesis under the same conditions. The study also implied a greater sensitivity of adipose-tissue lipogenesis, compared with hepatic or muscle lipogenesis, to inhibition by dietary fat. This *in vivo* effect of dietary fat was, however, not observed for fatty acid synthesis under the *in vitro* system except in subcutaneous adipose tissue. It is likely that there were differences in the hormonal environment between the *in vivo* and *in vitro* conditions, especially since insulin was used in the *in vitro* assay. Differences in sensitivity between peripheral and internal adipose tissue depots could be responsible for the differences in responses of mesenteric and perirenal adipose tissue to the dietary fat compared to the response of subcutaneous adipose tissue. The lipogenic response of adipose tissue *in vivo* was reflected by a significant decrease in enzymatic capacity of FAS. This decreased activity (Table 4.2) was probably due to a decrease in the mass of the FAS protein (Table 4.4) in the *de novo* fatty acid biosynthetic pathway.

In liver, Clarke and Jump (1992) showed that the ability of a dietary fat to regulate gene transcription was dependent on the carbon length of the fatty acid component, and also, on the location of double bonds within the fatty acids. As in liver,

lipogenesis and FAS gene expression in adipose tissue are regulated by hormonal and dietary factors (Mildner and Clarke 1991; Moustaid and Sul 1991). However, it is much less clear whether the changes in adipocyte lipogenesis observed actually reflected altered expression of genes coding for lipogenic enzymes because very few lipogenic genes have been cloned for study in domestic animals.

ACC protein abundance was reduced slightly in response to dietary fat supplementation. To the contrary, the catalytic activity of ACC was significantly elevated. This would not have been predicted from measurement of the enzyme protein abundance or the fatty acid synthetic rates. It is apparent that dietary fat regulates ACC and FAS differently in adipose tissues of sheep but the mechanisms that may be involved can not be identified by this study. However, one possible explanation for differences in response between ACC and FAS could be that ACC was less responsive to nutritional manipulation than is FAS. Moreover, as the fatty acids of adipose tissue mostly exist as esters of triacylglycerols, whose major fatty acids in ruminants are saturated or monounsaturated, the proportions of polyunsaturated fatty acids in the tissues are usually low. Consequently, the polyunsaturated fatty acid suppression of lipogenic enzyme expression may be weak in ruminant adipose tissue (Iritani et al. 1996) because of the relatively low concentrations.

6.3. Effect of Thermal Environments on the Regulation of Lipogenesis

There have been no previous direct measurements of the effect of environmental temperature exposure on the rates of fatty acid synthesis and regulation of key regulatory enzymes of lipogenesis in ruminant species. However, there is considerable indirect

evidence to suggest influences of environmental temperature in this regard. During periods of cold exposure, animals must generate heat from oxidation of nutrients and body reserves (Tsuda et al. 1984), but the contribution of each substrate to total heat production (HP), however, has not been fully documented in sheep exposed to thermoneutral and cold environments.

Studies by Graham et al. (1959) suggested that lipid metabolism accounted for most of the increased energy requirement of sheep in a cold environment. However, McKay (1974) showed that substantial increases also occur in glucose oxidation in cold exposed sheep. As reported in Chapter three, the intakes, HP and average daily gain of animals in the cold environment (aside from the pair-fed groups) were significantly higher than those of the animals maintained in the warm environment. This overall increase in energy intake and HP in the cold environment was probably associated with an enhancement of lipid metabolism in the adipose tissue. However, the increased rates of fatty acid synthesis observed in adipose tissue of the cold exposed sheep (Table 3.4) were not expected since increased HP in the cold is usually associated with fatty acid mobilization. The high capacities for triacylglycerol uptake and *de novo* fatty acid synthesis, together with low oxidative capacities in white adipose tissue (Herpin et al. 1987) may also suggest that there could be increased lipolytic rates to meet the energetic requirement of the fatty acid utilizing tissues such as muscle. The increased fatty acid synthesis rates in this experiment may therefore be indicative of increased lipid turnover rates in support of thermogenesis and high metabolic capacity for thermoregulation.

Cold exposure is known to cause an increase in endogenous plasma catecholamines in sheep (Christopherson et al. 1978). Catecholamine release is associated

partly with cardiovascular readjustments taking place to maximize HP and minimize heat loss (vasoconstriction), and partly with actions on thermogenesis (that is, mobilization of energy rich substrates for catabolism; Herpin et al. 1991). In some experiments, plasma concentrations of nonesterified fatty acids quickly increased after the onset of cold stress in sheep (Sano et al. 1995) and the levels remained elevated during more prolonged cold exposure (Young 1975; Sano et al. 1995). This probably implied that lipolysis was enhanced in the cold environment. To the contrary, the rates of fatty acid synthesis were increased in the cold exposed animals. This is in agreement with observations of Buckley and Rath (1987) and McCormack and Denton (1977) in other species. As shown by these authors, there was simultaneous fatty acid synthesis and oxidation in brown adipose tissue of cold exposed mice, and the increase in fatty acid synthesis was shown to be due in part to an increase in activity of ACC and FAS in the tissue.

In another study with mice, Trayhurn (1981) showed that fatty acid synthesis rates in all tissues except the liver were much higher in cold-acclimated mice than in the warm-acclimated animals. For adipose tissue, the synthesis rate in the cold acclimated mice was five to six times that in the warm acclimated group. The fact that in the study reported in Chapter three the rate of fatty acid synthesis was higher in the pairfed group (at least in subcutaneous and perirenal fat), showed that differences in food intake in the two environments alone was not the reason for the increased rates of lipogenesis, but perhaps a consequence of the requirement for substrates for thermogenesis.

In the last experiment (Chapter five) conducted to test the hypothesis that thermal environment and β-AR agonist regulate lipogenesis in sheep, the rate of fatty acid synthesis was also increased during exposure of the animals to a cold environment in the

β-AR agonist fed group compared to the warm. As shown in Chapters three and four, the increased rates of fatty acid synthesis observed during prolonged cold exposure were paralleled by increases in FAS activity but not ACC activity, and an increase in the protein abundance of the two enzymes. This indicated that the regulatory role of the thermal environment on fatty acid synthesis was due, in part, to its influence on the amount of enzyme protein and the catalytic efficiency with respect to FAS. The mechanisms by which cold exposure regulates enzyme activity and protein abundance in ruminant tissue in the current studies are not known but changes in plasma hormone levels in the animals may be involved.

During cold exposure ACC activity was decreased in subcutaneous and mesenteric adipose tissues but not in perirenal adipose tissue in studies presented in Chapters two (Table 2.1) and four (Table 4.1). In animals exposed to a thermoneutral environment and injected interperitoneally with noradrenaline to mimic effects of cold environmental exposure, Gibbins et al. (1985) showed that the noradrenaline induced inactivation of ACC in brown adipose tissue. A similar explanation might account for the responses observed in white adipose tissue of sheep in the current study. In another experiment *in vitro*, Haystead et al. (1990) showed that cyclic-AMP was involved in the inactivation of ACC by adrenaline but the inactivation did not result from direct phosphorylation of the enzyme by cyclic-AMP-dependent-protein kinase.

6.4. Adrenergic Regulation of Lipogenesis in Sheep

β-Adrenergic agonists are known as energy repartitioning and anabolic agents, and in animals these agents act via a β-AR-mediated mechanism to promote increased protein

accretion (Choo et al. 1992; Wheeler and Koohmaraie 1992; Dawson et al. 1993) while decreasing fat stores (Thornton et al. 1985; Warriss et al. 1989; Moloney et al. 1990; Zhang and Grieve 1995). Speculations about changes in carcass composition in response to dietary β-AR agonists usually revolve around observations made on muscle and adipose tissue *in vitro*. The general view of the effects of β-AR agonists on carcass fat and lipogenesis has been that increased lipolysis is induced by stimulation of the presynaptic sympathetic neurones innervating the adipose tissue. These effects would be expected to stimulate lipolysis and decrease lipogenesis.

Several studies have investigated the mechanisms of action of β-AR agonist on fat metabolism and in fact many of these studies have yielded contradictory results. Mersmann (1987) showed that the β-AR agonist, clenbuterol, stimulated lipolysis *in vivo*. Thornton et al. (1985) also showed that clenbuterol decreased the rates of lipogenesis and increased the rates of lipolysis and that this was mediated in part by the inactivation of ACC. To the contrary, Hu et al. (1988) showed that the rate of lipogenesis *in vitro* was increased in adipose tissue from cimaterol fed sheep in the presence and absence of insulin compared to the controls. The results from the current study with β-AR agonist fed sheep in the cold environment are consistent with this latter evidence. The increased rates of fatty acid synthesis in adipose tissue from β-agonist treated cold-acclimated sheep were also supported by results of Wilson (1989) who showed that the β-AR agonist BRL-26830 increased the rate of fatty acid synthesis in white adipose tissue in rats. This author suggested that the observed increase in fatty acid synthetic rate was probably mediated by an increase in circulating insulin level produced by the β-AR agent, since treatment of the animals with streptozotocin abolished the observed increase. In the warm

environment in the present study, the use of the β-AR agonist L-644,969 produced a response opposite to that which was seen in the cold environment. Thus the reduced rates of fatty acid synthesis in the warm environment indicate that the β-AR agonist may affect metabolic functions in the same adipose tissue in different ways depending on the environmental temperature.

The decreased rates of fatty acid synthesis caused by feeding a β-AR agonist in the warm environment were also associated with a corresponding decrease in the activity of ACC in all adipose tissues. However, the absence of any corresponding increase in the activity of ACC in adipose tissues from cold exposed sheep was unexpected, but this may suggest that the increase seen in the rates of fatty acid synthesis in the cold environment with agonist usage was mediated by another mechanism other than direct control of ACC in sheep. In general, feeding the β-agonist to sheep inhibited the *in vitro* rate of fatty acid synthesis in adipose tissue in the warm but not in the cold environment. These data, therefore, consistently mirrored the state of knowledge in the research literature about the effects of β-AR agonists producing increased or decreased rates of fatty acid synthesis in adipose tissues. The basis for these differential actions of β-agonist in adipose tissue from sheep in warm and cold environments is unclear and will require further investigation.

It should be pointed out that the foregoing discussion compares the β-AR agonist L-644,969 fed to sheep in the current study to the effect of several other β-AR agonists reported in the literature. The justification for this type of comparison is based on the knowledge that L-644,969 is a stereoisomer of ractopamine and these and several other β-agonists (e.g. cimaterol, clenbuterol, isoproterenol) utilized in earlier studies all belong to the phenethanolamine group of compounds. Even with this commonality, however, it

is possible for the physiological activity and the chemical and pharmacokinetic characteristics of this specific agonist (L-644,969) to be different from the others.

6.5. About the Research: Methodological Considerations and Justification

In the current study as described in Chapter three, the rate of *in vivo* fatty acid synthesis was measured by injecting each lamb with a tracer dose of sodium [^{14}C]acetate (~ 200 μCi) via a jugular catheter and each lamb was killed 1 h thereafter. Favarger (1965) had however, indicated that the use of labelled acetate may underestimate the quantitative lipogenic importance of certain tissues such as liver where large amounts of endogenous acetyl-CoA are produced. It is doubtful that the current study will suffer from this type of limitation since endogenous acetyl-CoA produced via mitochondrial reactions does not equilibrate with the cytosolic acetyl-CoA in the ruminant. This is because of the lack of ATP-citrate-lyase and malic enzyme in ruminant tissues, which would negate any quantitative importance of mitochondrial transfer to the cytosol (Hanson and Ballard 1967).

The standard assay utilized for measuring ACC activity in the current experiments measured the difference in acid stable products generated by CO_2 fixation in the presence and absence of exogenous acetyl-CoA. Such assays however, can be hindered by the presence of contaminating carboxylases (Thampy 1989), notably, propionyl-CoA carboxylase and pyruvate carboxylase. Propionyl-CoA carboxylase can catalyze the carboxylation of acetyl-CoA to give malonyl-CoA at 3/100 the rate it carboxylates propionyl-CoA (Barman 1969).

Acetyl-CoA carboxylase usually exists in active and inactive states and the proportions of the active state will depend on the phosphorylation and polymerization status of the enzyme. The formation of the active polymeric form of the enzyme is enhanced by citrate but is inhibited by fatty acyl-CoA thioesters or by carboxylation of the enzyme-bound biotin. In contrast to ACC however, propionyl-CoA carboxylase is not citrate dependent (Barman 1969). Thus when present in large amounts due to leakage from mitochondria, it could give an erroneously high value for ACC activity when assayed either in the absence of citrate or when citrate is present at low concentrations (0 to 0.3 mM) (Vavvas et al. 1997). Davies et al. (1982) suggested also that although citrate-independent activity in tissue extracts could be due to ACC, it could also be due to pyruvate carboxylase activity in the enzyme preparation. This is because freezing the tissue in liquid nitrogen followed by thawing and homogenization could lead to leakage of the enzyme from mitochondria. Since pyruvate carboxylase activity is activated by acetyl-CoA, any traces of pyruvate in the enzyme preparation could contribute to acetyl-CoA dependent incorporation of ^{14}C from $H^{14}CO_3$ into acid stable material. However, the pyruvate concentration in ovine adipose tissue is about 40 nmol / g tissue in adult sheep and if it remained unaltered during homogenization would give rise to only 1.5 μM in the assay (Robertson et al. 1982). This amount is small compared to the 1.06 mM acetyl-CoA employed in my assay procedure.

To avoid any complications of contamination by extraneous carboxylase leakage from mitochondria that could contribute to ACC activity, some precautionary steps were taken in the extraction and assay of ACC. Frozen samples were ground into powder under liquid nitrogen without being allowed to thaw, and the powder was quickly

weighed and homogenized in a buffer using 'flash homogenization' procedure (20 s at maximum setting and 4 °C). This procedure could help reduce or perhaps eliminate mitochondrial rupture. Also, because ACC was assayed in the presence of 10 mM citrate, the contribution by propionyl-CoA carboxylase to total activity, if any, was expected to be minimal. Furthermore, the preincubation of the enzyme extracts with citrate in all my studies and the addition of citrate to the ACC assay system should increase the activity of ACC relative to that of pyruvate carboxylase. It is still possible, even, with the precautions taken that a small increase in the activity of ACC in extracts from tissues may be due to pyruvate or propionyl-CoA carboxylase.

Apart from the steps taken discussed above, there appears to be other ways by which samples could be handled to avoid potential complications of contamination. In preparations, that are high in ACC activities, such complications could be avoided by eluting the sample homogenates through a column of Sephadex G-25 which had been equilibrated with 20 mM Tris-HCl buffer and 1.0 mM dithiothreitol to remove pyruvate and citrate (McNeillie et al. 1981; Okine and Arthur 1997). A major problem with this procedure however, is that it may result in a substantial dilution of extracts, and is very tedious and time consuming. This made it impracticable for my research purposes because of the large number of different samples that would have to have been eluted. Mreover, the method would be unsuitable for tissues like muscle and liver from sheep that have relatively low ACC activity.

Another aspect of the research that is worth noting is the fact that ACC and FAS activities reported in Chapters four and five were several fold higher than those reported in Chapter two using the same method of analysis. One reason for the

differences may relate to differences in hormonal milieu of the animals *in vivo*. Another reason could be that feeding high carbohydrate diets in the studies reported in Chapters four and five compared to alfalfa pellets that were fed in Chapter two could have contributed to increases in activities of the enzymes due to increased energy intake and increased plasma glucose or glucose precursors. The diets used in Chapter four contained 60 % barley, in Chapter five the concentrate diet (fed along with alfalfa pellets) contained 77 % barley grain and 10 % corn. The characteristics of the high barley concentrate diets in this study could have modified ovine adipose tissue metabolism and have an influence on the rates of fatty acid synthesis and activities of the two key regulatory enzymes measured in these studies. Recently, Okine and Arthur (1997) showed that increases in ACC and FAS activity occurred in adipose tissue from lambs in response to feeding a high concentrate barley-based diet compared to alfalfa pellets. These increased enzyme activities could be related to an increased propionate concentration and decreased acetate: propionate ratios and their consequent influence on insulin secretion (Buchanan-Smith et al. 1973). However, increases in lipogenic enzyme activities in adipose tissue from sheep fed the concentrate diet were not associated with increased rates of incorporation of [1-^{14}C]acetate into fatty acids (Okine and Arthur 1997).

6.6. Applications of Research Findings to Animal Production and Future Direction

Two important factors have influenced animal management practices. One is the impact of economic pressures to substantially and continually improve the efficiency of meat, milk and wool production, and the other is the need to meet consumers' demand for high quality ruminant products. In recent years, there has been strong preference toward fat-

modified dairy products and meat with less subcutaneous fat but possessing sufficient intramuscular fat to preserve overall palatability characteristics of tenderness and juiciness. In ruminant meat producing animals, most of the fat deposited as triacylglycerol arises via *de novo* fatty acid synthesis in adipose tissue (Vernon 1986), and the rate at which this is done may be affected by the thermal and nutritional environments to which the animal is exposed. The investigation of the control and regulation of ACC and FAS by environment, diet and adrenergic agent in this thesis therefore, should provide an important basic step toward understanding the regulation of fatty acid synthesis in tissues from sheep and other ruminant species in different management circumstances.

By making use of the information in the current research and comparing it with related information in the literature from ruminant and non ruminant species, it is possible to discern a pattern of differential responses of tissues to hormone (including adrenergic agents), and nutrient manipulation. Differences in activities and pattern of expression of the lipogenic enzymes in tissues can be utilized to direct the activities of these lipogenic enzymes in a tissue specific manner to regulate lipogenesis in the domestic animal. One enzyme that is highly inducible and that can be regulated in this manner is FAS. According to Clarke (1993), one approach is to develop a pharmacological screening assay that could select for drugs that inhibit FAS expression in a tissue specific manner, hence its activity and rate of fatty acid synthesis could be so regulated. Because the lipogenic enzymes are coordinately regulated, an inhibitor of FAS expression may also possess the capacity to suppress ACC.

Based on enzyme isolation, activity and immunoreactivity characterization two isoforms of ACC (265- and 280-kDa) were identified in *longissimus dorsi* muscle. These two isoforms can be coordinately or independently regulated *in vivo* in non-ruminant species. The differential synthesis of these isoforms and their response to hormone (adrenergic agent) should provide a compelling argument for the potential for these isoforms to be manipulated in the muscle of ruminants. These may provide some model systems for the dissection of the individual roles of ACC isoforms through which the mechanisms underlying their regulation of fat synthesis in the ruminant animal can be properly addressed.

In domestic animals, most of the known functions of β-adrenoceptors have been established through administration of agonist and antagonist drugs. However, public resistance to the use of phenethanolamine leanness-enhancing agents in livestock production is widespread partly because of consumer reluctance to consume animal products containing drug residues. Because of possible differences in oral potency of several of β-AR agonists and the residue that may be left in animal products after administration to animals, research should continue to determine safe tissue concentrations for chemical residues for all of β-AR agonists and by so doing it may be that safe and effective β-agonists could be developed for use in animal production for the regulation of fat deposition and protein accretion.

The present study suggests that regional differences exist among fat depots in their relative capacities to carry out lipogenesis. Understanding how these are regulated could suggest approaches to strategically enhance lipogenesis in certain tissues and suppress or reduce lipogenesis in others. For example, it may be feasible to enhance

intramuscular fat deposition (marbling) at will while minimizing total carcass fat accumulation (and save feed energy requirements). Excessive fat deposition is energetically costly, although minimal fat stores provide animals with important energy reserves that serve to buffer the animal against adverse nutritional and climatic condition. This research provides a basic foundation on which to build plans for manipulating fat deposition through genetic and management strategies.

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APPENDIX ONE

A1.1. ACC Assay Buffer

Stock items (A)	**Buffer (B)** vol. or proportion of **A**
0.5 M Tris-Acetate	20 µl
16.5 mg / ml BSA (essentially fatty acid free)	10 µl
70 mM ATP*	5 µl
43.44 µM β-mercaptoethanol	5 µl
66 mM Mg-Acetate	12.5 µl
132 mM potassium-citrate	12.5 µl
5 mM Acetyl-CoA*	35 µl

*ATP and acetyl-CoA are prepared fresh

Distilled deionized water	40 µl
Bicarbonate†	**15 µl**
Enzyme preparation	10 µl

†Bicarbonate in final incubation volume is prepared as follows:

Cold bicarbonate	10 µl of 300 mM stock
Hot bicarbonate	1 µl = 1µCi
DD water	4 µl
Total	**15** µl

15 µl bicarbonate aliquot is counted in liquid scintillation analyzer: = **X** dpm. (see below).

Prepare individual items in stock A separately. Prepare assay buffer (B) from stock items (A) in the proportions shown. Aliquot 100 µl of buffer B into each assay tube. Add 40 µl of dd-H_2O and 15 µl of bicarbonate to each assay tube.

Equilibrate reaction tubes to the temperature of the assay in a water bath. Start reaction by adding 10 µl enzyme extract. **Total assay volume per tube should be 165 µl including enzyme.** At 4 min stop reaction with 25 µl 70% perchloric acid.

Blank assay excluded acetyl-CoA and volume is replaced with equivalent amount of water. If more than 10 µl of enzyme preparation is used, adjust the volume of dd-H_2O as appropriate.

A1.2. Calculations of ACC activity from assay reactions

Molar concentration of bicarbonate in assay mixture

(10 µl x 300 mM) / 165 µl = 18.18 mM (18.18 µmol / ml).

$NaHCO_3$ amount in final incubation mixture is:
18.18 µmol ml^{-1} x 0.165 ml = 3 µmol

1 µCi of $NaH^{14}CO_3$ is added giving rise to 0.33 µCi / µmol.
Therefore, the specific radioactivity (**SA**) of the assay solution is 0.33 µCi / µmol $NaHCO_3$.

SA = 0.33 x **X** dpm / µmol where **X** = 1 µCi in each assay tube.

$= 10^{-3}$ x 0.33**X** dpm / nmol.

ACC assay reaction product counted in Packard Scintillation analyzer:

= **Y** dpm per 4 min reaction time.

Corrected for blanks = $\mathbf{Y_b}$ dpm per 4 min reaction time.

nmol $H^{14}CO_3^-$ incorporated into malonyl-CoA per minute is calculated as:

$\mathbf{Y_b}$ dpm / (**4** min x **SA**) = **"Z"** nmol / min.

ACC results are expressed as nmol $H^{14}CO_3.min^{-1}.mg\ protein^{-1}$ as follow:

[(**Z** nmol / min) / µg protein in assay tube] x 1000.

A1.3. Calculations of FAS activity

$$\Delta C = \Delta A / E$$

Where ΔC = change in concentration of NADPH, ΔA = change in absorbance and E = extinction coefficient of NADPH (E_{340} nm = 6.22 $mM^{-1}\ cm^{-1}$).

Example: In 1 ml assay volume, If **ΔA** of NADPH is 0.0139 /(min.cm): then,

ΔC = 0.0139 / 6.22 mM min^{-1}.

= 0.002234 mM min^{-1} or 2.234 µM min^{-1} or 2.234 µmol /L min^{-1}

FAS activity = 2.234 nmol / min.

Expressing FAS activity as nmol/min/mg protein:

= [(2.234 nmol min^{-1})/ µg protein in assay)] x 1000

Expressing FAS activity as nmol palmitate synthesized min^{-1} mg $protein^{-1}$
Divide previous step by 14.

Note: 14 nmol NADPH are oxidized in the synthesis of 1 nmol palmitate from acetyl-CoA and malonyl-CoA.

APPENDIX TWO

A2. WESTERN-BLOT ANALYSES

A2.1. Reagents for Gel-Electrophoresis

1.5 M Tris-HCl, pH 8.8

To 100 ml dd-H_2O add:
3.6878 g Tris-HCl
15.3797 g Tris base The pH should come right to 8.8.

0.5 M Tris-HCl, pH 6.8

7.0196 g Tris-HCl
0.6700 g Tris Base
Make to 100 ml with dd-H_2O; pH is about 7.2, adjust to 6.8 with 5 N HCl.

30 % Acrylamide

8.76 g acrylamide {GIBCO BRL Life Technologies, Grand Island, NY}
0.24 g bisacrylamide {GIBCO BRL Life Technologies}
In 30 ml dd-H_2O and store in a cool, dark place for up to 1 wk.

5 X Loading Buffer (prepare 8 ml)

50 % Glycerol	4 ml Glycerol
312.5 mM Tris-HCl, pH 6.8	1.7 ml 1.5 M Tris-HCl, pH 6.8
10 % SDS	0.8 g SDS
Bromophenol blue	0.2 g Bromophenol blue
25 % β-mercaptoethanol	2 ml β-mercaptoethanol

Aliquot into microfuge tubes and store at – 20 °C.

5 X Electrode Running Buffer

30.2 g Tris Base
144 g Glycine
10 g SDS In 2 L dd-H_2O. Working solution = 1X buffer.

A2.2. Reagents for Protein Transfer and Antibody Incubation

Towbin's Transfer Buffer pH 8.3

12.1 g Tris Base
57.6 g Glycine
800 ml methanol
2 ml 10 % SDS In 4 L dd-H_2O and store at 4 °C.

PBS, pH 7.4

32 g NaCl
0.8 g KCl
5.76 g Na_2HPO_4
0.88 g KH_2PO_4

In 4 L dd-H_2O. Adjust pH to 7.4 with conc. HCl.

TBS, pH 7.4

32 g NaCl
0.8 g KCl
12 g Tris Base

In 4 L dd-H_2O. Adjust pH to 7.4 with conc. HCl.

TBST

TBS / 0.05 % Tween 20 (50 µl Tween-20 per 100 ml TBS)

A2.3. Gel Casting

Required four glass plates (2 short and 2 long), four 1.5 mm spacers, and two 15- or 10-well 1.5 mm combs.
Rinse glass plates with absolute ethanol and air-dry. Setup gel casting apparatus for Mini-PROTEAN II gel unit (Bio-Rad Laboratories, Mississauga, ON) following manufacturer's recommendation.

The gel consists of two parts – 5 % separating gel and 4 % stacking gel.
Prepare the 5 % separating gel as follow (sufficient for 4 mini-gels):

22.4 ml distilled-deionized water
10.2 ml 1.5 M Tris-HCl, pH 8.8
6.6 ml 30 % acrylamide
400 µl 10 % SDS
400 µl 10 % ammonium persulfate (0.1 g/ ml)[1‡] (GIBCO BRL)
32 µl TEMED[‡] (GIBCO BRL)

Pour separating gel between the two glass plates leaving at least 1 cm from the top of the smaller glass plate.
Overlay gel with water saturated-isobutanol and allow 30 to 45 min for polymerization

Prepare 4 % stacking gel as follow

12.2 ml distilled-deionized water
5.0 ml 0.5 M Tris-HCl, pH 6.8
2.6 ml 30 % acrylamide
200 µl 10 % SDS
100 µl ammonium persulfate[1‡]
20 µl TEMED[‡]

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[1] Prepare the ammonium persulfate daily.
[‡] Add ammonium persulfate and TEMED just before pouring the gel.

Wash the alcohol off the top of the separating gel with water.

Pour stacking gel and insert a 15- or 10-well 1.5 mm comb.
Add more stacking gel as the gel shrinks around the comb using pasteur pippet, and ensure that each well is uniformly cast. Allow gel to polymerize for 30 min. Following polymerization, overlay the gel with water to prevent oxidation of the acrylamide.

A2.4. Sample preparation

Aliquot volume of samples corresponding to desired protein for loading into a microfuge tubes and add an amount of 5 X loading dye such that the final concentration of the dye is 1 X.

Prepare Rainbow High Molecular Weight Markers (Amersham International plc, Bukinghamshire, England) by combining 10 µl of the marker with 2.5 µl of 5 X loading dye in a microfuge tube.

Heat sample and marker tubes in a boiling water bath for 5 min to denature the protein and quickly cool the tubes on ice.

Collect the samples and marker at the bottom of the microfuge tubes by a short (5 s) centrifugation.

A2.5. Electrophoresis

Remove water from top of the gel and set up gel unit into its electrophoretic chamber. Fill the wells with 1 X electrode running buffer.

Load the volume of sample required for appropriate detection into each well.
Fill chamber with 1 X electrode running buffer sufficient to cover the electrodes and the bottom of the glass plates. Also, fill the reservoir between the two gels with the same buffer.
Run gel at 100 V for 2.5 h

A2.6. Protein Transfer to Membrane

After electrophoresis, mark the gel for orientation by nicking it in the bottom right corner and equilibrate gels in Towbin's buffer (4 °C) for 30 min on a shaker.

Presoak NitroPure membranes (Micron Separations Inc., Westborough, MA), fibre pads and 3 mm Whatmann chromatograhic paper in Towbin's buffer.

Setup a fibre pad and a 3 mm Whatman Chromatographic paper on the black surface of the gel-holder and roll out air bubble with a small glass test tube.

Sandwich gel next to nitrocellulose membrane in Towbin's buffer

Place the gel holder in the Mini Trans-Blot (Bio-Rad Laboratories) electrophoretic transfer system and perform transfer at 100 v for 2 h or overnight at 40 mA and 4 °C.

Following transfer, incubate membranes in Ponceau S stain (Sigma) for 5 min on a shaker to confirm transfer efficiency. Destain the membranes with distilled water.

The membranes may be stored at 4 °C if desired or proceed with antibody incubation.

A2.7. Antibody Incubation

Wash membranes in two changes of PBS for 5 min each.
Incubate membranes in 10 % skim milk / PBS / 0.1 % Tween-20 (*5 g skim milk and 50 µl Tween-20 in 50 ml PBS*) for 2 h to block non-specific binding.

Rinse membranes with TBST and wash in two changes of TBST for 5 min each.

Incubate membranes with appropriate antibody concentration (primary antibody in the case of FAS) for 1 h in 10 ml of solution (1 % skim milk in TBST).

After antibody incubation, wash membranes for 15 min in TBST, and in three more changes of TBST for 5 min each.

For FAS only, incubate membrans with appropriate concentration of secondary antibody for 1 h in TBST and wash as in the preceding step.

After antibody incubation is completed for either ACC or FAS, incubate each membrane in 6 ml of ECL detection reagent (i.e. 3 ml of Reagent 1 + 3 ml of Reagent 2; Amersham International plc, Bukinghamshire, England) for 1 min and place membrane in a plastic bag. Wipe out excess reagent.

Quickly place membrane in an x-ray cassette with Hyper-Film ECL and expose film to membrane for a length of time sufficient to generate a signal for analysis (15 to 25 s).

Develop the film and analyze the results using an imaging densitometer (Bio-Rad laboratories).

APPENDIX THREE

A.3. Effect of Environmental Temperature and Dietary Lipid Supplement on Fatty Acid Profile of Ovine Longissimus dorsi muscle, Liver and Adipose Tissues

A3.1. Fatty Acid Composition Analyses

The animal experiment was as described in Chapter three. Extraction of total lipids from tissue samples was done according to Folch et al. (1957) as described in Chapter three. For determination of fatty acid composition, methyl esters were prepared by using the technique of Metcalfe et al. (1966) as modified by Wijngaarden (1967). Two ml aliquots of Folch extracts from adipose tissue or 8-ml of feed, muscle or liver extracts were saponified at 100 °C for 1 h in 1 ml 0.5 N methanolic potassium hydroxide. After saponification, 1ml each of hexane and boron trifluoride were added and boiled at 100 °C for 1 h. After cooling to room temperature 1 ml hexane and 1 ml water were added to the mixture to extract the fatty acid methyl esters. After separation at room temperature, 100 µl aliquots of the supernatant were sampled into 1.5 ml GC vials with 1.2 ml hexane. Fatty acid composition of the esters was determined with a gas chromatograph (Varian Aerograph, series 3600, Sydney Australia) equipped with a flame ionization detector and helium as the carrier gas. Fatty acids were separated using a 30 m x 0.25 mm i.d. fused silica capillary columns (Supelco Canada, Ltd., Oakville ON). The oven was temperature-programmed from 180 to 230 °C at a rate of 10 °C per min. Separation of the fatty acid methyl esters occurred according to molecular weight and degree of unsaturation. Individual fatty acid methyl esters were identified by comparison of the peak retention times for the samples with those from reference standards. For each sample the area under each peak was quantified using a Shimadzu EZChrom software program (Shimadzu Scientific Instruments, Inc., Columbia, MD). All fatty acids are expressed as percentages by weight of total fatty acid measured in each sample.

A3.2. Statistical Analyses

Data were analyzed as a 3 x 2 factorial design using the GLM procedure (SAS 1996) and the models are as described in chapter three. In MS, period effect was significant for 15:0 ($p = 0.03$), 17:0 ($p = 0.01$) and 20:5ω3 ($p = 0.03$) fatty acids and this was tested against error means square while Environment, Diet and Environment x Diet effects were tested against period x environment x diet as the error term. Data are presented as least square means of main treatment effects together with their pooled standard errors. Probabilities less than .05 are considered to be different.

A3.3. Results

A3.3.1. Fatty Acid Composition of Tissues

The fatty acid composition of the two diets is shown in Table A3.1 and that of the tissues is shown in Tables A3.2 to A3.6. Relatively small effects of environment and dietary lipid supplementation on tissue fatty acid composition were observed. In *longissimus dorsi* muscle lipids (Table A3.2), there was no effect ($p > 0.05$) of environmental temperature on nearly all the fatty acids with the exception of 15:0 and 17:0. The proportions of these two fatty acids significantly increased in LD muscle from the cold cold-pairfed sheep compared to the warm environment. The proportions of saturated, unsaturated and polyunsaturated fatty acids in *longissimus dorsi* muscle were not affected by environment or diet.

In liver (Table A3.3), the proportion of 17:0 fatty acid was significantly increased ($p = 0.02$) in the cold environment. There was no effect of environment on the proportions of the remaining fatty acids. The proportions of saturated and unsaturated fatty acids were also not significantly affected. Significant effects of diet were observed for 15:0, 17:0, 18:1*tr* and 18:1ω9*cis* ($p < 0.05$) and 18:1ω7*cis* ($p = 0.05$). There was a decrease in the percentages of these fatty acids following lipid supplementation except for 18:1ω9*cis* that was increased.

In subcutaneous adipose tissue lipids (Table A3.4), the proportions of individual saturated fatty acids were not affected ($p > 0.05$) by environmental temperature. With unsaturated fatty acids, there was an increase in the proportion of 16:1ω7 in the cold pair-fed groups ($p = 0.004$), and also a tendency for an increase ($p = 0.07$) in the proportion of 20:5ω3 in the cold environment. Dietary lipid supplementation significantly decreased 15:0 ($p = 0.04$), 16:1ω7 ($p = 0.005$) and 17:0 ($p = 0.04$) concentrations in subcutaneous adipose tissue. Lipid supplementation significantly increased percentage of 18:0 fatty acid ($p = 0.002$) concentration but there was no effect of environment. The interaction of environment and diet, however, tended ($p= 0.06$) to influence 18:0. Although there was no significant effect of either environment or diet on 18:1ω7, the interaction of environment and diet on this fatty acid was significant ($p = 0.01$) with the result that fat supplementation decreased 18:1ω7 content in the cold environment but had the opposite effect in the warm environment.

Among the unsaturated fatty acids in mesenteric adipose tissue (Table A3.5), the percentage composition of 16:1ω7 was significantly increased ($p = 0.03$) in the cold compared to the warm environment. Percentage composition of 18:1*tr* was also numerically higher in the cold-pairfed (COPF) environment than in the warm environment. There was no significant effect of environment on the percentage composition of any of the individual saturated fatty acids. Feeding the lipid supplemented diet significantly increased ($p < 0.05$) the proportions of 16:0 in mesenteric adipose tissue. There was a significant decrease ($p = 0.02$) in 18:3ω3 and a tendency to decrease ($p= 0.07$) in 17:0, 18:1*tr* and 20:5ω3. No interactions of the main treatment effects were observed in fatty acids of mesenteric fat lipids.

In total lipid from perirenal adipose tissue (Table A3.6), the proportion of saturated fatty acids were not affected by exposure of the animals to various environments. With unsaturated fatty acids, cold exposure significantly reduced the proportions of 18:1ω7cis ($p = 0.004$). There was also a tendency for the cold environment to reduce 20:4ω6 ($p = 0.07$) and 20:5ω3 ($p = 0.08$).

Overall, there was no effect of diet on fatty acids from perirenal adipose tissue lipid, except with 16:0 that showed an elevation with dietary lipid supplementation ($p = 0.02$).

On average, LD muscle had relatively higher 16:0 content compared to the liver and subcutaneous fat, but the value was numerically similar to the 16:0 content of mesenteric and perirenal adipose tissue depots. The Percentage of 18:0 fatty acids in LD muscle in this study was similar to that observed in subcutaneous fat but lower than observed for 18:0 in mesenteric and perirenal adipose tissues. The proportions of 18:0 in the liver was the highest (~27 %) of all the tissues examined in this study.

In both muscle and liver, the trans- fatty acid 18:1*tr* was numerically lower than observed in all three adipose tissue depots. The proportion of monounsaturated fatty acid 18:1ω9 cis in LD muscle was about 36 %, and this amount was much higher than the 20 % in liver, and 29 % in subcutaneous, 23 % in mesenteric and 27 % in perirenal adipose tissue. The polyunsaturated fatty acid, 18:2ω6, was about 7 % in LD muscle. In liver, 18:2ω6 fatty acid constitutes 11 % of the total fatty acid profile, higher than the 4 % observed in adipose tissue depots.

In muscle, 18:3ω3, 20:4ω6 and 20:5ω3 altogether constitute about 2% of the total fatty acid profile. In liver, the proportion of the three fatty acids was higher (at least 6 %) whereas in adipose tissues, their contribution was less than 1 % of the total fatty acid profile.

A3.4. Discussion

This study consisted of a 3 x 2 factorial arrangement with the main effects of environment and diet on tissue fatty acid composition presented in Tables A3.2 to A3.6. Overall, 18:0, 18:1*trans*, 18:1ω9cis, 18:1ω7cis and 18:2 ω6 fatty acids constitute more than 80% of the fatty acids in all the tissues examined. Environment or diet did not affect the total amounts of saturated, unsaturated and polyunsaturated fatty acids. However, the proportion of unsaturated fatty acids

was higher in *longissimus dorsi* muscle and subcutaneous fat and lower in liver, and, mesenteric and perirenal fat depots compared to saturated fatty acids.

Results of this study also revealed some significant effects of environment and diet on several individual fatty acids. In muscle, liver and the three fat depots taken at slaughter, the percentage of 18:1 fatty acid in each tissue was significantly higher compared to 16:0 fatty acid although there were no significant treatment effects. The ratio of 16:1/18:0 in subcutaneous fat has been used as an index of unsaturation in Jersey cattle (Leat 1975). In the current experiment, the 16:1/18:0 ratio showed a general increase in the cold environment compared to the warm environment, indicating that cold exposure may promote some degree of unsaturation in sheep. On the contrary, there was no significant effect of diet on 16:1/18:0 ratio in all the tissues examined, except however, in subcutaneous adipose tissue where 16:1/18:0 ratio was reduced ($p < 0.05$) by lipid supplementation. When another index of unsaturation (total number of unsaturated bonds) was calculated and analyzed, there was no significant effect of environment or diet on these indices. However, between tissues, the unsaturation index was significantly higher for liver, followed by muscle and lowest in adipose tissue depots. Within the adipose tissue depots, the total number of unsaturated bonds was significantly higher ($p < 0.05$) in peripheral subcutaneous adipose tissue compared to the internal (mesenteric and perirenal) adipose tissue depots.

When overall means for subcutaneous, mesenteric and perirenal adipose tissues were considered, the primary difference in fatty acid composition between the three appeared to be in the relative proportions of 16:0, 18:0 and 18:1 fatty acids. The two internal adipose tissue depots contained significantly more 16:0 and 18:0 compared to peripheral subcutaneous fat depot. However, lipid extract from subcutaneous fat contained more 18:1 than mesenteric and perirenal fat suggesting that there could be more desaturase enzyme activity in subcutaneous fat.

These findings of small changes in fatty acid composition due to environmental temperature are in agreement with the results of Myres and Bowland (1973) that changes in fatty

acid composition in adipose tissue from pigs due to environmental temperature are negligible. The current observations, however, are in contrast to those of MacGrath et al. (1968) who showed a greater degree of back fat unsaturation in pigs exposed to a cold environment compared to those maintained in a warm environment. Moreover, in some other earlier studies, temperature and diet were shown to affect fatty acid composition in ruminants (Marchello et al. 1967; Leat 1977) with high ambient temperature resulting in increased saturation of fatty acids.

Under the condition of the study, there was little evidence that raising the lambs in a cold environment or feeding the lipid supplement produced compositional changes that would be beneficial in a major way for human health. It was possible that the relatively small changes in tissue fatty acid composition observed in the current study might be due to the fact that 5 wk of temperature acclimation or diet feeding might not have been sufficient to exert a large impact on tissue fatty acid composition. It is also possible that the limited effect observed in fatty acid composition could be due to the relatively small difference in fat composition of the diets fed to the animals.

In consideration of the increased ratios of 16:1/18:0 observed in the cold environment, the implications and significance of these ratios in sheep have yet to be determined. The role of environmental temperature especially a cold environment in this regard is not clear. It is possible that a cold environment may act as a stimulation to enhance a dehydrogenase enzyme system, which would encourage the biosynthesis of more unsaturated fat (Marchello et al. 1967). A potential advantage of this may be to maintain the fluidity of the peripheral subcutaneous adipose tissue membrane at cooler temperatures.

Table A3.1. Fatty acid composition (% weight of total fatty acids) of control and lipid supplemented diets

Fatty acids	Control diet	Lipid diet
14:0	0.97	1.18
15:0	0.12	0.16
16:0	29.98	20.73
16:1ω7	0.17	0.35
17:0	0.12	0.08
18:0	3.95	2.40
18:1*tr*	Not detected	0.25
18:1ω9*cis*	35.28	45.74
18:1ω7*cis*	1.68	1.80
18:2ω6	25.73	23.75
18:3ω3	2.00	3.56
Total SFA[z]	35.14	24.55
Total UFA[y]	64.86	75.45

[z]Total saturated fatty acids

[y]Total unsaturated fatty acids

Table A3.2. Effect of cold exposure and dietary lipid supplementation of sheep on fatty acid composition (% of total fatty acids) of *longissimus dorsi* muscle of sheep.

Fatty-acids	Environment (E)[z]					Diet (D)[y]				E*D[x]
	CO	COPF	WA	SE	Prob.	CTL	LIPID	SE	Prob.	
14:0	2.67	2.43	2.34	0.16	0.32	2.54	2.42	0.13	0.64	0.09
15:0	0.31[b]	0.42[a]	0.33[b]	0.03	0.03	0.38	0.34	0.02	0.19	0.73
16:0	26.84	24.94	26.03	0.08	0.27	26.24	25.63	0.66	0.51	0.24
16:1ω7	1.87	2.06	1.92	0.14	0.61	1.97	1.92	0.11	0.75	0.24
17:0	1.24[b]	1.61[a]	1.31[ab]	0.10	0.04	1.47	1.29	0.08	0.12	0.67
18:0	14.53	14.79	15.55	0.65	0.48	15.24	14.68	0.52	0.42	0.71
18:1*tr*	3.11	4.01	3.47	0.50	0.49	3.42	3.64	0.40	0.73	0.71
18:1ω9*cis*	39.23	36.04	34.34	2.59	0.37	35.83	37.25	2.06	0.59	0.81
18:1ω7*cis*	2.20	2.42	7.62	1.59	0.47	3.98	2.18	1.27	0.30	0.44
18:2ω6	6.08	8.36	7.13	0.79	0.14	6.55	7.83	0.63	0.16	0.20
18:3ω3	0.70	0.99	1.15	0.22	0.31	0.85	1.05	0.18	0.40	.086
20:4ω6	0.92	1.40	1.55	0.28	0.25	1.11	1.47	0.23	0.27	0.77
20:5ω3	0.30	0.53	0.26	0.15	0.31	0.43	0.30	0.12	0.50	0.58
Total SFA[w]	45.59	43.90	45.56	1.45	0.69	45.87	44.16	1.16	0.31	0.38
Total UFA[v]	54.41	56.10	54.44	1.45	0.69	54.13	55.84	1.16	0.31	0.38
Total PUFA[u]	8.00	11.16	10.10	1.12	0.13	8.93	10.57	0.90	0.20	0.56
U-Index[t]	65.85	72.55	69.58	2.05	0.11	67.40	71.25	1.76	0.13	0.43

Values are least square means of main treatment effects. N = 8 for environment and 12 for diet.

[z]CO = cold environment, COPF = cold pair-fed, and, WA = warm environment.

[y]CTL, LIPID are respectively control or lipid supplemented diet.

[x]E*D = Interaction of environment and diet. SE = standard error of least square means.

[a, b] For individual fatty acids, means within same treatment effect (environment or diet) and followed by different superscripts are different ($p < 0.05$).

[w]SFA = saturated fatty acids; [v]UFA = unsaturated fatty acids; [u]PUFA = polyunsaturated fatty acids.

[t]U-Index = Index of total number of unsaturated bonds.

Table A3.3. Effect of environmental temperature and dietary lipid supplementation on fatty acid composition (% of total fatty acids) of sheep liver.

Fatty-acids	Environment (E)[y]					Diet (D)[x]				E*D[w]
	CO	COPF	WA	SE	Prob.	CTL	LIPID	SE	Prob.	
14:0	0.86	0.74	0.74	0.08	0.44	0.74	0.82	0.06	0.41	0.35
15:0	0.43	0.43	0.50	0.05	0.46	0.54[e]	0.36[f]	0.04	0.004	0.44
16:0	21.40	20.50	22.51	1.00	0.38	20.54	22.40	0.82	0.13	0.75
16:1ω7	1.43	1.43	1.27	0.12	0.55	1.43	1.33	0.10	0.48	0.87
17:0	2.28[ab]	2.72[a]	1.89[b]	0.19	0.02	2.62[e]	1.98[f]	0.15	0.01	0.26
18:0	27.31	26.83	28.05	1.32	0.81	27.22	27.57	1.07	0.82	0.74
18:1*tr*	5.18	4.75	4.13	0.51	0.36	5.59[e]	3.78[f]	0.42	0.01	0.02
18:1ω9*cis*	20.61	19.69	18.64	0.82	0.26	18.11[e]	21.18[e]	0.67	0.005	0.18
18:1ω7*cis*	2.63	3.77	2.42	0.54	0.20	3.52	2.36	0.44	0.08	0.47
18:2ω6	10.38	12.13	11.44	1.37	0.67	11.00	11.62	1.12	0.70	0.25
18:3ω3	0.93	0.65	1.58	0.30	0.10	1.23	0.87	0.24	0.31	0.66
20:4ω6	5.33	5.05	5.70	0.90	0.88	6.17	4.56	0.74	0.14	0.95
20:5ω3	1.23	1.31	1.12	0.09	0.32	1.28	1.16	0.07	0.27	0.95
Total SFA[w]	52.28	51.22	53.69	1.99	0.68	51.66	53.13	1.63	0.53	0.59
Total UFA[v]	47.72	48.78	46.30	1.99	0.68	48.34	46.87	1.63	0.53	0.59
Total PUFA[u]	17.87	19.14	19.84	2.04	0.79	19.68	18.22	1.66	0.54	0.53
U-Index[t]	80.87	82.60	82.50	6.40	0.98	85.42	78.56	5.22	0.37	0.77

Values are least square means of main treatment effects. N = 8 for environment and 12 for diet.

[z]CO = cold environment, COPF = cold pair-fed, and, WA = warm environment.

[y]CTL, LIPID are respectively control or lipid supplemented diet.

[x]E*D = Interaction of environment and diet. SE = standard error of least square means.

[a, b] For individual fatty acids, means within same treatment effect (environment or diet) and followed by different superscripts are different ($p < 0.05$).

[w]SFA = saturated fatty acids; [v]UFA = unsaturated fatty acids; [u]PUFA = polyunsaturated fatty acids.

[t]U-Index = Index of total number of unsaturated bonds.

Table A3.4. Effect of cold exposure and dietary lipid supplementation of sheep on fatty acid composition (% of total fatty acids) of subcutaneous adipose tissue.

Fatty-acids	Environment (E)[z]					Diet (D)[y]				E*D[x]
	CO	COPF	WA	SE	Prob.	CTL	LIPID	SE	Prob.	
14:0	2.69	2.78	2.57	0.24	0.83	2.88	2.48	0.19	0.16	0.93
15:0	1.31	1.094	0.91	0.15	0.20	1.30[e]	0.91[f]	0.12	0.04	0.64
16:0	24.14	23.80	24.44	0.91	0.89	23.47	24.79	0.75	0.23	0.29
16:1ω7	1.80[b]	2.06[a]	1.64[b]	0.08	0.004	2.03[e]	1.64[f]	0.06	0.01	0.10
17:0	4.31	3.60	3.24	0.05	0.34	4.39[e]	3.05[f]	0.41	0.04	0.76
18:0	14.13	13.79	15.52	0.71	0.11	12.80[f]	15.7[e]	0.58	0.002	0.06
18:1*tr*	6.13	7.92	6.28	0.61	0.10	6.72	6.83	0.50	0.88	0.35
18:1ω9*cis*	29.67	28.72	28.10	2.48	0.90	29.73	27.93	2.03	0.54	0.15
18:1ω7*cis*	11.10	11.81	12.52	1.78	0.85	11.71	11.90	1.45	0.93	0.01
18:2ω6	3.90	4.25	4.14	0.38	0.80	4.17	4.02	0.31	0.75	0.43
18:3ω3	0.63	0.56	0.58	0.12	0.92	0.63	0.55	0.10	0.58	0.98
20:4ω6	0.11	0.11	0.11	0.02	0.94	0.11	0.11	0.01	0.91	0.12
20:5ω3	0.10	0.09	0.07	0.01	0.07	0.09	0.08	0.01	0.22	0.30
Total SFA[w]	46.58	44.47	46.57	1.22	0.39	44.81	46.93	1.00	0.15	0.66
Total UFA[v]	53.42	55.53	53.43	1.22	0.39	55.19	53.07	1.00	0.15	0.66
Total PUFA[u]	4.73	5.01	4.90	0.50	0.92	4.99	4.76	0.41	0.69	0.61
U-Index[t]	59.27	61.60	59.32	1.54	0.49	61.30	58.83	1.26	0.18	0.76

Values are least square means of main treatment effects. N = 8 for environment and 12 for diet.

[z]CO = cold environment, COPF = cold pair-fed, and, WA = warm environment.

[y]CTL, LIPID are respectively control or lipid supplemented diet.

[x]E*D = Interaction of environment and diet. SE = standard error of least square means.

[a, b] For individual fatty acids, means within same treatment effect (environment or diet) and followed by different superscripts are different ($p < 0.05$).

[w]SFA = saturated fatty acids; [v]UFA = unsaturated fatty acids; [u]PUFA = polyunsaturated fatty acids.

[t]U-Index = Index of total number of unsaturated bonds.

Table A3.5. Effect of cold exposure and dietary lipid supplementation on fatty acid composition (% of total fatty acids) of mesenteric adipose tissue from sheep.

Fatty-acids	Environment (E)[z]					Diet (D)[y]				E*D[x]
	CO	COPF	WA	SE	Prob.	CTL	LIPID	SE	Prob.	
14:0	3.46	3.27	3.21	0.27	0.67	3.39	3.23	0.17	0.52	0.19
15:0	0.60	0.61	0.57	0.04	0.74	0.63	0.56	0.03	0.21	0.83
16:0	27.72	27.77	29.41	1.14	0.28	26.28[f]	29.66[e]	0.93	0.02	0.55
16:1ω7	1.27[ab]	1.47[a]	1.16[b]	0.08	0.03	1.34	1.26	0.06	0.40	0.46
17:0	2.37	2.56	1.94	0.19	0.10	2.51	2.07	0.16	0.07	0.85
18:0	23.04	20.57	22.09	1.63	0.57	22.81	20.99	1.33	0.35	0.53
18:1*tr*	6.80	8.93	7.02	0.62	0.05	8.25	6.92	0.51	0.07	0.23
18:1ω9*cis*	22.31	23.66	24.50	2.28	0.79	22.90	24.08	1.86	0.66	0.53
18:1ω7*cis*	8.10	6.67	4.58	2.06	0.49	6.23	6.67	1.68	0.85	0.58
18:2ω6	3.54	4.54	4.61	0.40	0.13	4.61	3.85	0.33	0.12	0.43
18:3ω3	0.61	0.73	0.72	0.08	0.49	0.85[e]	0.54[f]	0.06	0.002	0.14
20:4ω6	0.10	0.11	0.11	0.02	0.78	0.12	0.09	0.01	0.25	0.79
20:5ω3	0.09	0.11	0.09	0.01	0.24	0.10	0.08	0.01	0.07	0.30
Total SFA[w]	57.18	53.78	57.22	1.55	0.23	55.61	56.51	1.27	0.62	0.46
Total UFA[v]	42.82	46.22	42.78	1.55	0.23	44.39	43.49	1.27	0.62	0.46
Total PUFA[u]	4.34	5.49	5.53	0.49	0.17	5.68	4.56	0.40	0.06	0.39
U-Index[t]	48.23	52.99	49.51	1.79	0.18	51.46	49.03	1.46	0.26	0.35

Values are least square means of main treatment effects. N = 8 for environment and 12 for diet.

[z]CO = cold environment, COPF = cold pair-fed, and, WA = warm environment.

[y]CTL, LIPID are respectively control or lipid supplemented diet.

[x]E*D = Interaction of environment and diet. SE = standard error of least square means.

[a, b]For individual fatty acids, means within same treatment effect (environment or diet) and followed by different superscripts are different ($p < 0.05$).

[w]SFA = saturated fatty acids; [v]UFA = unsaturated fatty acids; [u]PUFA = polyunsaturated fatty acids.

[t]U-Index = Index of total number of unsaturated bonds.

Table A3.6. Effect of temperature and dietary lipid supplementation on fatty acid composition (% of total fatty acids) of perirenal adipose tissue from sheep.

Fatty-acids	Environment (E)[z]					Diet (D)[y]				E*D[x]
	CO	COPF	WA	SE	Prob.	CTL	LIPID	SE	Prob.	
14:0	3.16	2.97	2.73	0.28	0.53	2.93	2.99	0.23	0.74	0.22
15:0	0.42	0.42	0.45	0.06	0.90	0.45	0.42	0.05	0.57	0.29
16:0	27.69	26.19	27.15	1.56	0.85	24.10[f]	29.93[e]	1.24	0.003	0.15
16:1ω7	1.07	1.23	1.24	0.15	0.65	1.25	1.10	0.12	0.45	0.62
17:0	2.42	2.16	2.02	0.25	0.45	2.35	2.05	0.20	0.29	0.59
18:0	24.57	23.91	20.55	2.75	0.52	24.53	21.51	2.19	0.33	0.99
18:1*tr*	6.88	7.86	9.60	2.63	0.73	9.65	6.57	2.09	0.26	0.19
18:1ω9*cis*	27.60	28.26	24.96	1.97	0.43	27.20	26.68	1.57	0.81	0.99
18:1ω7*cis*	1.68[b]	2.26[ab]	6.19[a]	1.26	0.04	2.98	3.77	1.08	0.57	0.52
18:2ω6	3.76	4.13	4.44	0.48	0.56	3.90	4.32	0.38	0.49	0.84
18:3ω3	0.68	0.50	0.55	0.12	0.49	0.58	0.58	0.10	0.93	0.50
20:4ω6	0.04	0.04	0.06	0.01	0.07	0.05	0.04	0.01	0.22	0.56
20:5ω3	0.03	0.04	0.05	0.01	0.08	0.05	0.04	0.01	0.15	0.68
Total SFA[w]	58.27	55.68	52.91	2.95	0.40	54.35	56.88	2.34	0.42	0.54
Total UFA[v]	41.73	44.32	47.09	2.95	0.40	45.65	43.12	2.34	0.42	0.54
Total PUFA[u]	4.51	4.71	5.11	0.57	0.72	4.57	4.98	0.45	0.51	0.78
U-Index[t]	47.09	49.74	53.02	3.03	0.35	51.04	48.86	2.42	0.52	0.53

Values are least square means of main treatment effects. N = 8 for environment and 12 for diet.

[z]CO = cold environment, COPF = cold pair-fed, and, WA = warm environment.

[y]CTL, LIPID are respectively control or lipid supplemented diet.

[x]E*D = Interaction of environment and diet. SE = standard error of least square means.

[a, b] For individual fatty acids, means within same treatment effect (environment or diet) and followed by different superscripts are different ($p < 0.05$).

[w]SFA = saturated fatty acids; [v]UFA = unsaturated fatty acids; [u]PUFA = polyunsaturated fatty acids.

[t]U-Index = Index of total number of unsaturated bonds.

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